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Vol. 1

Genome and Disease

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Vol. 1

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Contents

VII **Preface**

1 **The Genomic Basis of Disease, Mechanisms and Assays for Genomic Disorders**
Stankiewicz, P.; Lupski, J.R. (Houston, Tex.)

17 **Gross Deletions and Translocations in Human Genetic Disease**
Abeysinghe, S.S.; Chuzhanova, N.; Cooper, D.N. (Cardiff)

35 **Nucleotide Excision Repair and Related Human Diseases**
Bergoglio, V.; Magnaldo, T. (Villejuif)

53 **Oxidative Damage to DNA in Non-Malignant Disease: Biomarker or Biohazard?**
Evans, M.D.; Cooke, M.S. (Leicester)

67 **Dominant Non-Coding Repeat Expansions in Human Disease**
Dick, K.A.; Margolis, J.M.; Day, J.W.; Ranum, L.P.W. (Minneapolis, Minn.)

84 **Telomeres and Telomerase in Stem Cells during Aging and Disease**
Ju, Z.; Rudolph, K.L. (Hannover)

104 **Retrotransposable Elements and Human Disease**
Callinan, P.A.; Batzer, M.A. (Baton Rouge, La.)

116 **The Spindle Checkpoint and Chromosomal Stability**
Qi, W.; Yu, H. (Dallas, Tex.)

131 Protein Kinases That Regulate Chromosome Stability and Their Downstream Targets
Nojima, H. (Osaka)

149 The Role of the APC Tumor Suppressor in Chromosomal Instability
Alberici, P.; Fodde, R. (Rotterdam)

171 c-Myc, Genomic Instability and Disease
Kuttler, F.; Mai, S. (Winnipeg)

191 Nijmegen Breakage Syndrome and Functions of the Responsible Protein, NBS1
Antoccia, A. (Rome); Kobayashi, J. (Kyoto); Tauchi, H. (Mito); Matsuura, S. (Hiroshima); Komatsu, K. (Kyoto)

206 Werner Syndrome, Aging and Cancer
Ozgenc, A.; Loeb, L.A. (Seattle, Wash.)

218 Fanconi Anemia: Causes and Consequences of Genetic Instability
Kalb, R.; Neveling, K.; Nanda, I.; Schindler, D.; Hoehn, H. (Würzburg)

243 Author Index
244 Subject Index

Preface

The first volume of the new book series *Genome Dynamics* is dedicated to 'Genome Instability and Human Disease'. Cancer and other genetic human diseases are caused by a variety of mutations ranging from subtle sequence changes to larger genomic rearrangements and alterations in chromosome number and structure. With contributions of reputed experts, this book aims to update our knowledge of the multiple mechanisms of genomic instability leading to human disease. Emphasis is given to the different types of genomic sequences involved in disease-related genomic rearrangements, as well as to the various exogenous factors increasing the frequency of mutations. Several chapters are dedicated to the dysfunction of important cellular mechanisms like DNA repair and chromosome segregation, which leads to genomic instability and generally to tumorigenesis. Important 'caretaker' genes controlling the stability of our genome have been identified through their defects in genomic instability syndromes, which are also extensively reviewed in this volume.

All papers published in *Genome Dynamics* are reviewed according to classical standards. I would like to thank all contributors and referees involved in this special issue, Dr. Michael Schmid and his team for their invaluable help during the preparation of this volume, as well as Dr. Thomas Karger for giving us the opportunity to launch this book series.

Jean-Nicolas Volff
Würzburg, March 2006

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The Genomic Basis of Disease, Mechanisms and Assays for Genomic Disorders

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Abstract

In the past fifteen years, an emerging group of genetic diseases have been described that result from DNA rearrangements rather than from single nucleotide changes. Such conditions have been referred to as genomic disorders. The predominant molecular mechanism underlying the rearrangements that cause this group of diseases and traits is nonallelic homologous recombination (NAHR) (unequal crossing-over between chromatids or chromosomes) utilizing low-copy repeats (LCRs) (also known as segmental duplications) as substrates. In contradistinction to highly repetitive sequences (e.g. *Alu* and LINE elements), these higher-order genomic architectural features usually span >1 kb and up to hundreds of kilobases of genomic DNA, share >96% sequence identity and constitute >5% of the human genome. Many LCRs have complex structure and have arisen during primate speciation as a result of serial segmental duplications. LCRs can stimulate and/or mediate constitutional (both recurrent and nonrecurrent), evolutionary, and somatic rearrangements. Recently, copy-number variations (CNVs), also referred to as large-scale copy-number variations (LCVs) or copy-number polymorphisms (CNPs), parenthetically often associated with LCRs, have been demonstrated as a source of human variation as well as a potential cause of diseases. In addition to fluorescence in situ hybridization (FISH), pulsed-field gel electrophoresis (PFGE), and *in silico* analyses, multiplex ligation-dependent probe amplification (MLPA), and array comparative genomic hybridization (aCGH) with BAC and PAC clones have proven to be useful diagnostic methods for the detection and characterization of DNA rearrangements with the latter enabling high-resolution genome-wide analysis. The clinical implementation of such techniques is revolutionizing clinical cytogenetics.

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The concept of genomic disorders refers to recurrent and usually submicroscopic DNA rearrangements involving unstable genomic regions [1]. The major

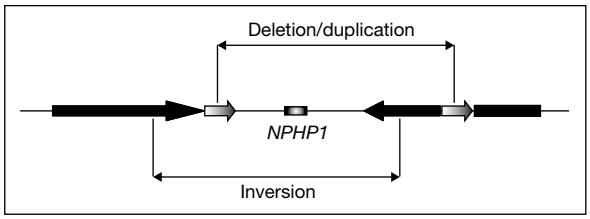

Fig. 1. LCR-based complex DNA structure. Genomic architecture of the familial juvenile nephronophthisis (NPHP1) chromosome region at 2q13. NAHR between inverted ~300-kb LCRs can lead to non-pathogenic submicroscopic inversion found in heterozygous status in ~21% of control individuals and homozygous status in 1.3% of normal individuals. Only 45-kb subunits are oriented in the same directions stimulating and mediating the recurrent ~290-kb deletion identified in homozygous state in ~80% of patients with recessive NPHP1 [15] and likely polymorphic reciprocal duplication (Cheung and Beaudet, unpublished observation) involving the dosage sensitive *NPHP1* gene.

molecular genetic mechanism responsible for these events is an intra- or inter-chromosomal nonallelic homologous recombination (NAHR), or unequal crossing-over, between low-copy repeats (LCRs) [2–9]. LCRs also known as segmental duplications, duplicons, or paralogous sequences are genomic fragments of high sequence identity (>96%), range in size between >1 kb and hundreds of kilobases and account for ~5% of the human genome [10–13]. When located in direct orientation, LCRs can mediate deletions or reciprocal duplications of the genomic region located between them, whereas NAHR between inverted LCRs can result in inversion of the intervening genomic segment [1]. In the LCRs of a more complex structure consisting of both direct and inverted subunits, distinct subunits can serve as NAHR substrates, leading to either inversion or a deletion/duplication, depending on the orientation of those utilized as the recombination substrates (fig. 1) [1, 14–18].

Genomic disorders are caused in most cases by de novo rearrangements that occur with a high $\sim 10^{-4}$ frequency [14]. The abnormal phenotype results from a deletion, duplication, or disruption of the dosage-sensitive gene(s) contained in the rearranged region. Based on the LCR/NAHR mechanism, equal frequencies of the recurrent common deletions and the reciprocal duplications are expected. Similar to large chromosomal segmental aneusomies yielding partial monosomies, that are known to manifest with the more severe phenotype than the reciprocal trisomies, the submicroscopic deletions may potentially be associated with an increased prenatal lethality relative to their reciprocal duplications. This could theoretically result in a higher prevalence of the postnatal duplications. However, probably because of the ascertainment bias due to the milder or even

normal phenotype, and the molecular diagnostic challenge of identifying 3:2 vs. 2:1 dosage differences, only a few cases of reciprocal duplications have been described to date. Some of this frequency discrepancy may disappear as more subjects will be analyzed using better screening methods.

Moreover, the same frequencies of genomic disorders might be anticipated in different world populations if genome architecture is similar among different people. However, the observation of significant differences in frequencies for one common deletion causing Sotos syndrome, ranging from ~9% in the European population to 50% in Japan [19–24], suggests a continuous evolution of LCRs.

Evolution of LCRs

Most of the LCRs responsible for disease associated rearrangements are not present in the mouse genome and have arisen recently, during primate speciation [17]. *Alu-Alu* recombination has been proposed to have played an important role in the origin of many of them [25–27].

The level of sequence identity between LCRs is thought to reflect the time of their origin during evolution. The later the LCRs arose, the higher the sequence identity between them is expected. However, ongoing gene conversion events have led to the homogenization of LCRs and thus inaccuracy in dating events using current molecular clock analysis. For example, molecular and computational studies of the LCRs in 17p revealed evidence for continuous recombination events between ~115 kb LCR17pA/D and ~118 kb LCR17pD copies of 98.5% sequence identity in patients with Smith-Magenis syndrome (SMS) and an uncommon but recurrent deletion [28]. Conversely, no deletions have been identified between LCR17pA/C (~79 kb) and LCR17pC (~91 kb) copies that are thought to have arisen evolutionarily at the same time as LCR17pA/D and LCR17pD, yet have only ~88% sequence identity. This suggests that the deficiency in gene conversion events between the latter copies resulted in declining frequency of NAHR with divergence decreasing homology to below the recombination stimulating threshold and subsequent lack of recombination events [29].

Recombination Hotspots

The strand exchanges for NAHR sites are not scattered throughout the length of homology within LCRs, but cluster in recombination hotspots [3, 30]. The hotspots were localized to a 500-bp region within the ~24-kb CMT1A-REPs

[30–32], 2 kb in NF1-REPs [33], 1.1 kb with 501-bp stretch of perfect identity within ~200-kb SMS-REPs [34], 524 bp within the ~120-kb LCR17pA and LCR17pD [28] and 2.5–3 kb in ~50–65 kb Sos-REPs [18, 35]. However, no specific primary or secondary DNA sequence motifs or features of DNA in these hotspots could be identified [36].

Proximal 17p

The proximal chromosome 17p, in which >23% of the genomic sequence consists of LCRs, has provided an excellent model for studying the role of genome architecture involving LCRs in the origin of genomic disorders with constitutional, evolutionary, and somatic rearrangements having been described [37].

CMT1A/HNPP

Charcot-Marie-Tooth type 1 disease (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP) are well known genomic disorders caused in >99% of cases by copy-number change of a dosage sensitive gene *PMP22* as a result of reciprocal duplication or deletion of a ~1.4-Mb genomic fragment within 17p12, respectively [3, 38]. This genomic segment is flanked by two ~24-kb and ~98.7% identical LCRs, termed the proximal CMT1A-REP and the distal CMT1A-REP, which serve as substrates for NAHR [2, 39].

SMS/dup(17)(p11.2p11.2) Syndrome

SMS is a multiple congenital anomalies and mental retardation disorder resulting from haploinsufficiency of the *RAI1* gene on chromosome 17p11.2 [40–44]. In 70–80% of SMS patients, *RAI1* is deleted along with an ~4-Mb genomic segment flanked by large, complex, highly identical (~98.7%), and directly oriented, proximal (~256 kb) and distal (~176 kb) LCRs termed SMS-REPs via the NAHR mechanism (common deletion) [45, 46]. A third LCR copy, the middle SMS-REP (~241 kb) is inverted and located between the proximal and distal copies [46]. In about 4% of the SMS patients, an uncommon but recurrent microdeletion ~5 Mb in size was found to be mediated by NAHR utilizing the directly oriented LCR17pA/D and LCR17pD copies [28, 47]. The remainder (20–25%) of SMS patients with chromosome deletions harbor nonrecurrent unusual smaller and larger sized deletions; the majority of which have LCRs at both or one breakpoint, indicating the important role of genomic architecture in their formation [28, 34, 47–49]. Analysis of products of recombination in nonrecurrent unusual sized SMS deletions

revealed nonhomologous end joining (NHEJ) as an important underlying mechanism [50].

Using molecular and computational analyses of the human and primate genomic regions, the complex structure and evolution of LCRs in proximal 17 p was delineated. This complex genome architecture was found to have arisen over the course of primate speciation during the interval of ~3–50 million years ago, as a result of serial segmental duplication events [29, 46].

Somatic Rearrangements

The burden of the evolutionary legacy of LCRs in proximal 17 p was shown to be responsible also for the recurrent somatic rearrangement, isodicentric chromosome idic(17)(p11). This isodicentric chromosome is frequently found in various hematological malignancies including chronic myeloid leukemia (CML) and in solid tumors such as childhood primitive neuroectodermal tumors (PNETs) and signifies a poor prognosis. The idic(17)(p11) breakpoints cluster within five cruciform structures containing ~38–49-kb LCRs of ~99.8% identity in the SMS common deletion region [51].

Genomic architecture involving LCRs has been shown to also play an important role in the formation of the most frequent chromosome abnormalities found in chronic myeloid leukemia – Philadelphia chromosome translocation t(9;22)(q34;q11) [52]. Recently, DNA cruciform structures have been found to be causative for gene amplifications in cancer [53].

Microduplications

Dup(17)(p11.2p11.2) – The Homologous Recombination Reciprocal of the Common SMS Deletion

The ~4-Mb genomic interval commonly deleted in patients with SMS has been found to be duplicated in patients with dup(17)(p11.2p11.2). This duplication causes a relatively mild and variable physical and behavioral phenotype including dysmorphic craniofacial features, aortic root enlargement, hypotonia, failure to thrive, oropharyngeal dysphagia, neurocognitive impairment, autistic, aggressive, and self-injurious behavior and sleep disturbances; the latter distinct from those in patients with common deletion 17p11.2 [54]. The degree of organ dysgenesis also appears to be less prominent with the duplication in comparison to the deletion.

Dup(22)(q11.2q11.2)

The role of LCR/NAHR has been shown also in the origin of a dup(22)(q11.2q11.2) syndrome. However, unlike the dup(17)(p11.2p11.2) syndrome, the sizes of the duplications vary with distal breakpoints mapping to different

distal LCR22s and only a portion of them being reciprocal to the common 3-Mb deletion found in patients with DiGeorge/Velocardiofacial (DGS/VCFS) syndrome. This likely suggests that larger duplications are more tolerated than larger deletions [4, 55, 56]. The phenotype of the dup(22)(q11.2q11.2) syndrome is extremely diverse [57] and only in a few patients does it resemble the DGS/VCFS features [55]. LCR22s mediate also the formation of chromosome inv dup(22) associated with cat eye syndrome as well as the most frequent non-Robertsonian recurrent translocation t(11;22)(q23;q11.2) [58].

Dup(15)(q11.2q13)

Unequal crossing-over between LCRs on chromosome 15 leads to rare interstitial duplications and triplications and to the more common inv dup(15). Maternally derived interstitial duplications of 15q11.2-q13 are associated with various types of intellectual impairment, including autistic spectrum disorders [59, 60], that are distinct from the reciprocal Prader-Willi (PWS) and Angelman (AS) deletion syndromes. Paternal interstitial duplications and inv dup(15) have been described very rarely and are most likely benign.

To date, the predicted duplication reciprocal to the Williams-Beuren syndrome (WBS) deletion and other deletion syndromes caused by the LCR/NAHR mechanism, remain to be identified.

Submicroscopic Inversions

Submicroscopic paracentric inversions due to NAHR between inverted LCRs, can manifest with an abnormal phenotype when one of the breakpoints disrupts the haploinsufficient gene. The first such cases described involved the *F8* gene in ~50% of patients with hemophilia A [61], the *IDS* gene in 13% of patients with Hunter disease [62], and the *EMD* gene on Xq28 in some patients with Emery-Dreifuss muscular dystrophy [63, 64].

The LCRs associated with WBS have a complex structure with some subunits positioned in direct and others in inverted orientation [65]. The analysis of the WBS region in parents of the patients with WBS revealed that about one third of parents transmitting the deleted chromosome are a carrier of a constitutional inversion between the LCR subunits oriented in opposite direction [9, 66]. Using *cis*-morphisms [67] (paralogous sequence variants, site specific nucleotide differences, or multisite variations), Bayés et al. [9] proposed that WBSinv-1 inversion occurs as an intrachromosomal rearrangement utilizing most likely the middle and telomeric subunits B of WBS-LCRs, followed by interchromosomal rearrangements, leading to genomic deletion. Interestingly,

the WBSinv-1 has been identified also in three individuals with clinical features that show some similarity to WBS [66].

A heterozygous inversion with breakpoints in PWS/AS-LCRs BP2 and BP3 has been identified in four of six mothers of children with AS due to the chromosome deletions and was not found in mothers of AS patients with paternal uniparental disomy 15. Furthermore, this inversion was also found in 9% of 44 controls [68]. However, to date, no genomic inversions in 15q11.2q13 have been described in fathers of patients with PWS.

More than 80% of nucleotide sequences that constitute LCR22-2 and LCR22-4 in the DGS chromosome region on 22q11.2 are in direct orientation. No genomic inversions have been identified between LCR22-2 and LCR22-4 [69, 70]. Similarly, a search for potential inversions revealed no rearrangements between inversely oriented middle SMS-REP and either proximal or distal SMS-REP copies in the parents of children with SMS caused by 17p11.2 deletion (Stankiewicz and Lupski, unpublished observation).

Studies on normal individuals revealed that 26% of the European population and 39% of the Japanese population carry a submicroscopic 3.5-Mb heterozygous paracentric 8p23.1 inversion with breakpoints within the olfactory receptor LCRs [16, 71]. The inverted abnormal chromosome was demonstrated to be responsible for the origin of the frequent recurrent inv dup(8p) chromosome [16] as well as recurrent translocation t(4;8)(p16;p23) [72].

Similar to the recurrent t(4;8)(p16;p23) translocation, Jobling et al. [73] showed that the 3-Mb inversion in Yp present in some males can stimulate the formation of the *PRKX/PRKY* translocation responsible for most XX males and some XY females. Furthermore, cryptic inversions via the complex series of deletions and duplications mediated by several types of LCRs result in azoospermia c (AZFc) [74, 75].

Recently, Stefansson et al. [76] identified a ~900-kb inversion polymorphism in about 20% of Europeans which was rare in Africans and almost absent in East Asians. This inversion is associated with a haplotype block. Interestingly, individuals carrying this inversion have more children and have higher recombination rates than noncarriers.

Copy-Number Variations (CNVs)

Recent genome-wide screenings using array comparative genomic hybridization (aCGH) with BAC clones and representational oligonucleotide microarray analysis (ROMA) have identified hundreds of apparent copy-number variations (CNVs), alternatively referred to as large-scale copy-number variations

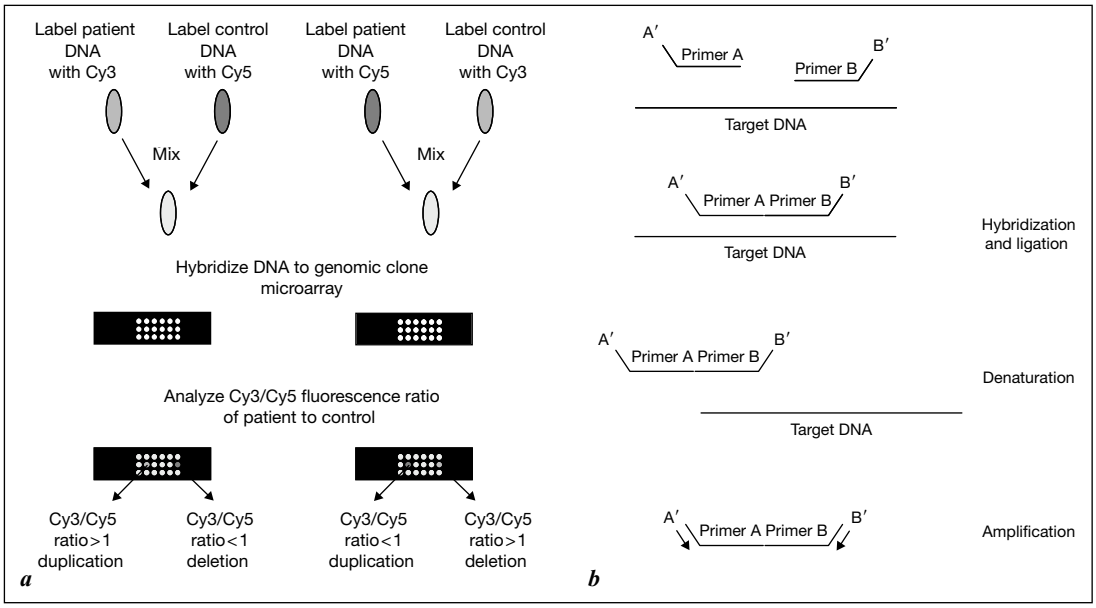

Fig. 2. Principle of aCGH and MLPA. ***a*** In contrast to conventional CGH where metaphase chromosome spreads are used as a template for the hybridization reaction, in aCGH, the arrays of BAC or PAC clone DNAs are spotted on the glass slide. The following steps are similar to conventional CGH. The analyzed and reference DNAs are labeled differentially and co-hybridized usually in duplicate. The gain or loss of copy-number is reflected by deviation of the yellow color signal present when there is a 1:1 ratio of both DNAs. ***b*** In the MLPA technique, the A and B PCR primers specific for the adjacent DNA fragments of the analyzed genomic region are labeled with A′ and B′ unique sequence that will be used subsequently for the PCR amplification. After the hybridization step, both primers are ligated and later denatured from the target DNA. The A′ and B′ fragments of the ligation product serve as a template for the PCR reaction and reflect the copy-number of the analyzed locus. Both techniques are sensitive enough to detect 1.5:1 duplication ratio changes.

(LCVs) or copy-number polymorphisms (CNPs), among apparently normal individuals [77–79]. These rearrangements ranged in size between 100 kb to 2 Mb (average 300–460 kb), encompassed genes and were associated in several cases with LCRs. In support of these findings, recently, Sharp et al. [80] demonstrated that LCRs are a major stimulator of large-scale variation in the human genome. Using *in silico* analysis, they identified 130 potential rearrangement hotspots flanked by LCRs and by a custom aCGH with over 2,000 BAC clones they identified 119 CNPs, 73 of which were previously unreported. The extent to which copy-number variation occurs among different world populations, and to what extent they may convey either Mendelian or complex traits, remains to be determined.

Methods for Analysis of Genomic Disorders

For many years the detection of large >30 kb yet submicroscopic (<2–5 Mb and beyond the level of resolution of conventional G-banding) rearrangements associated with genomic disorders required pulsed-field gel electrophoresis (PFGE) and fluorescence in situ hybridization (FISH) techniques to resolve genomic changes of such magnitude [37, 67]. The public availability of the human genomic DNA sequence as a result of the efforts of the Human Genome Project enabled the computational analysis of genomic regions and has been an important adjunct for PFGE and FISH; however, these technologies are still limited to the examination of specific genomic regions. Recently, progress in the development of aCGH has enabled high-resolution screening of the entire genome simultaneously.

Array CGH

The replacement of metaphase chromosomes as targets in conventional CGH with BAC or PAC (bacterial or P1 artificial chromosome) clones immobilized as arrays on glass slides enables detection of copy-number changes throughout the entire human genome with a much better resolution (~150 kb vs. ~5–10 Mb) (fig. 2a) [81, 82]. The level of resolution is essentially without limit, depending only on the size and distance between the arrayed interrogating probes. aCGH has been applied successfully for the analysis of chromosome regions [83–85], whole chromosomes [86], and entire genomes in a single test [87–90]. Recently, aCGH proved also to be a powerful and promising method supplementing current routine diagnostic procedures in clinical cytogenetics [91–93]. Different kinds of target DNAs including cDNAs [94], PCR products [95], and oligonucleotides [96, 97] have been used successfully.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

As an alternative approach for genome-wide screening for the detection of specific large deletions or duplications in genomic DNA, a quantitative PCR-based technique called multiplex ligation-dependent probe amplification (MLPA) has been applied [98] (fig. 2b). This method is a refinement of the multiplex amplifiable probe hybridization (MAPH) approach [99] that has been used for screening of subtelomeric chromosome abnormalities [100–102].

Conclusions

Recent application of novel and promising genome-wide screening techniques, aCGH, and MLPA that enable detection of submicroscopic unbalanced

genomic rearrangements together with intensive *in silico* analysis of the publicly available DNA sequence from the Human Genome Project have demonstrated a previously underappreciated role for genomic rearrangements as a cause of genetic diseases. The results of intensive studies of genomic architecture in unstable chromosome regions, such as proximal 17 p, elucidated LCR/NAHR and NHEJ as major molecular mechanisms responsible for recurrent and nonrecurrent genomic rearrangement, respectively. Depending on the gene content, genome architecture-catalyzed rearrangements can manifest with frequent well known genomic disorders or be associated with CNVs whose phenotypic consequences remain to be determined.

References

1 Lupski JR: Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. Trends Genet 1998;14:417–422.
2 Pentao L, Wise CA, Chinault AC, Patel PI, Lupski JR: Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1.5 Mb monomer unit. Nat Genet 1992;2:292–300.
3 Reiter LT, Murakami T, Koeuth T, Pentao L, Muzny D, Gibbs RA, Lupski JR: A recombination hotspot responsible for two inherited peripheral neuropathies is located near a *mariner* transposon-like element. Nat Genet 1996;12:288–297. Correction in Nat Genet 1998;19:303.
4 Edelmann L, Pandita RK, Morrow BE: Low-copy repeats mediate the common 3-Mb deletion in patients with velo-cardio-facial syndrome. Am J Hum Genet 1999;64:1076–1086.
5 Amos-Landgraf JM, Ji Y, Gottlieb W, Depinet T, Wandstrat AE, Cassidy SB, Driscoll DJ, Rogan PK, Schwartz S, Nicholls RD: Chromosome breakage in the Prader-Willi and Angelman syndromes involves recombination between large, transcribed repeats at proximal and distal breakpoints. Am J Hum Genet 1999;65:370–386.
6 Dorschner MO, Sybert VP, Weaver M, Pletcher BA, Stephens K: *NF1* microdeletion breakpoints are clustered at flanking repetitive sequences. Hum Mol Genet 2000;9:35–46.
7 Shaw CJ, Bi W, Lupski JR: Genetic proof of unequal meiotic crossovers in reciprocal deletion and duplication of 17p11.2. Am J Hum Genet 2002;71:1072–1081.
8 Stankiewicz P, Lupski JR: Genome architecture, rearrangements and genomic disorders. Trends Genet 2002;18:74–82.
9 Bayés M, Magano LF, Rivera N, Flores R, Pérez Jurado LA: Mutational mechanisms of Williams-Beuren syndrome deletions. Am J Hum Genet 2003;73:131–151.
10 Cheung VG, Nowak N, Jang W, Kirsch IR, Zhao S, Chen X-N, Furey TS, Kim U-J, Kuo W-L, Olivier M, Conroy J, Kasprzyk A, Massa H, Yonescu R, Sait S, Thoreen C, Snijders A, Lemyre E, Bailey JA, Bruzel A, Burrill WD, Clegg SM, Collins S, Dhami P, Friedman C, Han CS, Herrick S, Lee J, Ligon AH, Lowry S, Morley M, Narasimhan S, Osoegawa K, Peng Z, Plajzer-Frick I, Quade BJ, Scott D, Sirotkin K, Thorpe AA, Gray JW, Hudson J, Pinkel D, Ried T, Rowen L, Shen-Ong GL, Strausberg RL, Birney E, Callen DF, Cheng J-F, Cox DR, Doggett NA, Carter NP, Eichler EE, Haussler D, Korenberg JR, Morton CC, Albertson D, Schuler G, de Jong PJ, Trask BJ: Integration of cytogenetic landmarks into the draft sequence of the human genome. Nature 2001;409:953–958.
11 Bailey JA, Yavor AM, Massa HF, Trask BJ, Eichler EE: Segmental duplications: Organization and impact within the current human genome project assembly. Genome Res 2001;11:1005–1017.
12 Cheung J, Estivill X, Khaja R, MacDonald JR, Lau K, Tsui LC, Scherer SW: Genome-wide detection of segmental duplications and potential assembly errors in the human genome sequence. Genome Biol 2003;4:R25.

13 She X, Jiang Z, Clark RA, Liu G, Cheng Z, Tuzun E, Church DM, Sutton G, Halpern AL, Eichler EE: Shotgun sequence assembly and recent segmental duplications within the human genome. Nature 2004;431:927–930.
14 Shaffer LG, Lupski JR: Molecular mechanisms for constitutional chromosomal rearrangements in humans. Annu Rev Genet 2000;34:297–329.
15 Saunier S, Calado J, Benessy F, Silbermann F, Heilig R, Weissenbach J, Antignac C: Characterization of the *NPHP1* locus: mutational mechanism involved in deletions in familial juvenile nephronophthisis. Am J Hum Genet 2000;66:778–789.
16 Giglio S, Broman KW, Matsumoto N, Calvari V, Gimelli G, Neumann T, Ohashi H, Voullaire L, Larizza D, Giorda R, Weber JL, Ledbetter DH, Zuffardi O: Olfactory receptor-gene clusters, genomic-inversion polymorphisms, and common chromosome rearrangements. Am J Hum Genet 2001;68:874–883.
17 Stankiewicz P, Lupski JR: Molecular-evolutionary mechanisms for genomic disorders. Curr Opin Genet Dev 2002;12:312–319.
18 Kurotaki N, Stankiewicz P, Wakui K, Niikawa N, Lupski JR: Sotos syndrome common deletion is mediated by directly oriented subunits within inverted Sos-REP low-copy repeats. Hum Mol Genet 2005;14:532–542.
19 Kurotaki N, Imaizumi K, Harada N, Masuno M, Kondoh T, Nagai T, Ohashi H, Naritomi K, Tsukahara M, Makita Y, Sugimoto T, Sonoda T, Hasegawa T, Chinen Y, Tomita Ha HA, Kinoshita A, Mizuguchi T, Yoshiura K, Ohta T, Kishino T, Fukushima Y, Niikawa N, Matsumoto N: Haploinsufficiency of *NSD1* causes Sotos syndrome. Nat Genet 2002;30:365–366.
20 Douglas J, Hanks S, Temple IK, Davies S, Murray A, Upadhyaya M, Tomkins S, Hughes HE, Cole TRP, Rahman N: *NSD1* mutations are the major cause of Sotos syndrome and occur in some cases of Weaver syndrome but are rare in other overgrowth phenotypes. Am J Hum Genet 2003;72:132–143.
21 Rio M, Clech L, Amiel J, Faivre L, Lyonnet S, Le Merrer M, Odent S, Lacombe D, Edery P, Brauner R, Raoul O, Gosset P, Prieur M, Vekemans M, Munnich A, Colleaux L, Cormier-Daire V: Spectrum of *NSD1* mutations in Sotos and Weaver syndromes. J Med Genet 2003;40:436–440.
22 Kurotaki N, Harada N, Shimokawa O, Miyake N, Kawame H, Uetake K, Makita Y, Kondoh T, Ogata T, Hasegawa T, Nagai T, Ozaki T, Touyama M, Shenhav R, Ohashi H, Medne L, Shiihara T, Ohtsu S, Kato Z, Okamoto N, Nishimoto J, Lev D, Miyoshi Y, Ishikiriyama S, Sonoda T, Sakazume S, Fukushima Y, Kurosawa K, Cheng JF, Yoshiura K, Ohta T, Kishino T, Niikawa N, Matsumoto N: Fifty microdeletions among 112 cases of Sotos syndrome: low copy repeats possibly mediate the common deletion. Hum Mutat 2003;22:378–387.
23 Kamimura J, Endo Y, Kurotaki N, Kinoshita A, Miyake N, Shimokawa O, Harada N, Visser R, Ohashi H, Miyakawa K, Gerritsen J, Innes AM, Lagace L, Frydman M, Okamoto N, Puttinger R, Raskin S, Resic B, Culic V, Yoshiura K, Ohta T, Kishino T, Ishikawa M, Niikawa N, Matsumoto N: Identification of eight novel *NSD1* mutations in Sotos syndrome. J Med Genet 2003;40e:126.
24 Türkmen S, Gillessen-Kaesbach G, Meinecke P, Albrecht B, Neumann LM, Hesse V, Palanduz S, Balg S, Majewski F, Fuchs S, Zschieschang P, Greiwe M, Mennicke K, Kreuz FR, Dehmel HJ, Rodeck B, Kunze J, Tinschert S, Mundlos S, Horn D: Mutations in *NSD1* are responsible for Sotos syndrome, but are not a frequent finding in other overgrowth phenotypes. Eur J Hum Genet 2003;11:858–865.
25 Bailey JA, Liu G, Eichler EE: An *Alu* transposition model for the origin and expansion of human segmental duplications. Am J Hum Genet 2003;73:823–834.
26 Babcock M, Pavlicek A, Spiteri E, Kashork CD, Ioshikhes I, Shaffer LG, Jurka J, Morrow BE: Shuffling of genes within low-copy repeats on 22q11 (LCR22) by Alu-mediated recombination events during evolution. Genome Res 2003;13:2519–2532.
27 Zhou Y, Mishra B: Quantifying the mechanisms for segmental duplications in mammalian genomes by statistical analysis and modeling. Proc Natl Acad Sci USA 2005;102:4051–4056.
28 Shaw CJ, Withers MA, Lupski JR: Uncommon deletions of the Smith-Magenis syndrome region can be recurrent when alternate low-copy repeats act as homologous recombination substrates. Am J Hum Genet 2004;75:75–81.

29 Stankiewicz P, Shaw CJ, Withers M, Inoue K, Lupski JR: Serial segmental duplications during primate evolution result in complex human genome architecture. Genome Res 2004;14: 2209–2220.
30 Reiter LT, Hastings PJ, Nelis E, De Jonghe P, Van Broeckhoven C, Lupski JR: Human meiotic recombination products revealed by sequencing a hotspot for homologous strand exchange in multiple HNPP deletion patients. Am J Hum Genet 1998;62:1023–1033.
31 Lopes J, Ravise N, Vandenberghe A, Palau F, Ionasescu V, Mayer M, Levy N, Wood N, Tachi N, Bouche P, Latour P, Ruberg M, Brice A, LeGuern E: Fine mapping of de novo CMT1A and HNPP rearrangements within CMT1A-REPs evidences two distinct sex-dependent mechanisms and candidate sequences involved in recombination. Hum Mol Genet 1998;7:141–148.
32 Lopes J, Tardieu S, Silander K, Blair I, Vandenberghe A, Palau F, Ruberg M, Brice A, LeGuern E: Homologous DNA exchanges in humans can be explained by the yeast double-strand break repair model: a study of 17p11.2 rearrangements associated with CMT1A and HNPP. Hum Mol Genet 1999;8:2285–2292.
33 López-Correa C, Dorschner M, Brems H, Lázaro C, Clementi M, Upadhyaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns J-P, Marynen P, Stephens K, Legius E: Recombination hotspot in *NF1* microdeletion patients. Hum Mol Genet 2001;10:1387–1392.
34 Bi W, Yan J, Stankiewicz P, Park S-S, Walz K, Boerkoel CF, Potocki L, Shaffer LG, Devriendt K, Nowaczyk MJM, Inoue K, Lupski JR: Genes in a refined Smith-Magenis syndrome critical deletion interval on chromosome 17p11.2 and the syntenic region of mouse. Genome Res 2002;12:713–728.
35 Visser R, Shimokawa O, Harada N, Kinoshita A, Ohta T, Niikawa N, Matsumoto N: Identification of a 3.0-kb major recombination hotspot in patients with Sotos syndrome who carry a common 1.9-Mb microdeletion. Am J Hum Genet 2005;76:52–67.
36 Lupski JR: Hotspots of homologous recombination in the human genome: not all homologous sequences are equal. Genome Biol 2004;5:242.
37 Stankiewicz P, Inoue K, Bi W, Walz W, Park S-S, Kurotaki N, Shaw CJ, Fonseca P, Yan J, Lee JA, Khajavi M, Lupski JR: Genomic disorders – genome architecture results in susceptibility to DNA rearrangements causing common human traits. Cold Spring Harb Symp Quant Biol 2003;LXVIII: 445–454.
38 Chance PF, Abbas N, Lensch MW, Pentao L, Roa BB, Patel PI, Lupski JR: Two autosomal dominant neuropathies result from reciprocal DNA duplication/deletion of a region on chromosome 17. Hum Mol Genet 1994;3:223–228.
39 Reiter LT, Murakami T, Koeuth T, Gibbs RA, Lupski JR: The human *COX10* gene is disrupted during homologous recombination between the 24 kb proximal and distal CMT1A-REPs. Hum Mol Genet 1997;6:1595–1603.
40 Smith AC, McGavran L, Robinson J, Waldstein G, Macfarlane J, Zonona J, Reiss J, Lahr M, Allen L, Magenis E: Interstitial deletion of (17)(p11.2p11.2) in nine patients. Am J Med Genet 1986;24:393–414.
41 Stratton RF, Dobyns WB, Greenberg F, DeSana JB, Moore C, Fidone G, Runge GH, Feldman P, Sekhon GS, Pauli RM, Ledbetter DH: Interstitial deletion of (17)(p11.2p11.2): report of six additional patients with a new chromosome deletion syndrome. Am J Med Genet 1986;24: 421–432.
42 Greenberg F, Guzzetta V, Montes de Oca-Luna R, Magenis RE, Smith ACM, Richter SF, Kondo I, Dobyns WB, Patel PI, Lupski JR: Molecular analysis of the Smith-Magenis syndrome: a possible contiguous-gene syndrome associated with del(17)(p11.2). Am J Hum Genet 1991;49:1207–1218.
43 Chen K-S, Potocki L, Lupski JR: The Smith-Magenis syndrome [del(17)p11.2]: Clinical review and molecular advances. Ment Retard Dev Disabil Res Rev 1996;2:122–129.
44 Slager RE, Newton TL, Vlangos CN, Finucane B, Elsea SH: Mutations in *RAI1* associated with Smith-Magenis syndrome. Nat Genet 2003;33:466–468.
45 Chen K-S, Manian P, Koeuth T, Potocki L, Zhao Q, Chinault AC, Lee CC, Lupski JR: Homologous recombination of a flanking repeat gene cluster is a mechanism for a common contiguous gene deletion syndrome. Nat Genet 1997;17:154–163.
46 Park S-S, Stankiewicz P, Bi W, Shaw C, Lehoczky J, Dewar K, Birren B, Lupski JR: Structure and evolution of the Smith-Magenis syndrome repeat gene clusters, SMS-REPs. Genome Res 2002;12:729–738.

47 Stankiewicz P, Shaw CJ, Dapper JD, Wakui K, Shaffer LG, Withers M, Elizondo L, Park S-S, Lupski JR: Genome architecture catalyzes nonrecurrent chromosomal rearrangements. Am J Hum Genet 2003;72:1101–1116.
48 Trask BJ, Mefford H, van den Engh G, Massa HF, Juyal RC, Potocki L, Finucane B, Abuelo DN, Witt DR, Magenis E, Baldini A, Greenberg F, Lupski JR, Patel PI: Quantification by flow cytometry of chromosome-17 deletions in Smith-Magenis syndrome patients. Hum Genet 1996;98: 710–718.
49 Vlangos CN, Yim DK, Elsea SH: Refinement of the Smith-Magenis syndrome critical region to approximately 950 kb and assessment of 17p11.2 deletions. Are all deletions created equally? Mol Genet Metab 2003;79:134–141.
50 Shaw CJ, Lupski JR: Non-recurrent 17p11.2 deletions are generated by homologous and non-homologous mechanisms. Hum Genet 2005;116:1–7.
51 Barbouti A, Stankiewicz P, Nusbaum C, Cuomo C, Cook A, Höglund M, Johansson B, Hagemeijer A, Park S-S, Mitelman F, Lupski JR, Fioretos T: The breakpoint region of the most common isochromosome, i(17q), in human neoplasia is characterized by a 220 kb region containing palindromic low-copy repeats. Am J Hum Genet 2004;74:1–10.
52 Saglio G, Storlazzi CT, Giugliano E, Surace C, Anelli L, Rege-Cambrin G, Zagaria A, Jimenez Velasco A, Heiniger A, Scaravaglio P, Torres Gomez A, Roman Gomez J, Archidiacono N, Banfi S, Rocchi MA: A 76-kb duplicon maps close to the BCR gene on chromosome 22 and the ABL gene on chromosome 9: possible involvement in the genesis of the Philadelphia chromosome translocation. Proc Natl Acad Sci USA 2002;99:9882–9887.
53 Tanaka H, Bergstrom DA, Yao MC, Tapscott SJ: Widespread and nonrandom distribution of DNA palindromes in cancer cells provides a structural platform for subsequent gene amplification. Nat Genet 2005;37:320–327.
54 Potocki L, Chen K-S, Park S-S, Osterholm DE, Withers MA, Kimonis V, Summers AM, Meschino WS, Anyane-Yeboa K, Kashork CD, Shaffer LG, Lupski JR: Molecular mechanism for duplication 17p11.2 – the homologous recombination reciprocal of the Smith-Magenis microdeletion. Nat Genet 2000;24:84–87.
55 Ensenauer RE, Adeyinka A, Flynn HC, Michels VV, Lindor NM, Dawson DB, Thorland EC, Lorentz CP, Goldstein JL, McDonald MT, Smith WE, Simon-Fayard E, Alexander AA, Kulharya AS, Ketterling RP, Clark RD, Jalal SM: Microduplication 22q11.2, an emerging syndrome, clinical, cytogenetic, and molecular analysis of thirteen patients. Am J Hum Genet 2003;73: 1027–1040.
56 Hassed SJ, Hopcus-Niccum D, Zhang L, Li S, Mulvihill JJ: A new genomic duplication syndrome complementary to the velocardiofacial (22q11 deletion) syndrome. Clin Genet 2004;65:400–404.
57 Yobb TM, Somerville MJ, Willatt L, Firth HV, Harrison K, Mackenzie J, Gallo N, Morrow BE, Shaffer LG, Babcock M, Chernos J, Bernier F, Sprysak K, Christiansen J, Haase S, Elyas B, Lilley M, Bamforth S, McDermid HE: Microduplication and triplication of 22q11.2: a highly variable syndrome. Am J Hum Genet 2005;76:865–876.
58 McDermid HE, Morrow BE: Genomic disorders on 22q11. Am J Hum Genet 2002;70:1077–1088.
59 Cook EH Jr, Lindgren V, Leventhal BL, Courchesne R, Lincoln A, Shulman C, Lord C, Courchesne E: Autism or atypical autism in maternally but not paternally derived proximal 15q duplication. Am J Hum Genet 1997;60:928–934.
60 Bolton PF, Dennis NR, Browne CE, Thomas NS, Veltman MW, Thompson RJ, Jacobs P: The phenotypic manifestations of interstitial duplications of proximal 15q with special reference to the autistic spectrum disorders. Am J Med Genet 2001;105:675–685.
61 Lakich D, Kazazian HH Jr, Antonarakis SE, Gitschier J: Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. Nat Genet 1993;5:236–241.
62 Bondeson ML, Dahl N, Malmgren H, Kleijer WJ, Tonnesen T, Carlberg BM, Pettersson U: Inversion of the *IDS* gene resulting from recombination with *IDS*-related sequences is a common cause of the Hunter syndrome. Hum Mol Genet 1995;4:615–621.
63 Small K, Iber J, Warren ST: Emerin deletion reveals a common X-chromosome inversion mediated by inverted repeats. Nat Genet 1997;16:96–99.
64 Small K, Warren ST: Emerin deletions occurring on both Xq28 inversion backgrounds. Hum Mol Genet 1998;7:135–139.

65 Peoples R, Franke Y, Wang YK, Perez-Jurado L, Paperna T, Cisco M, Francke U: A physical map, including a BAC/PAC clone contig, of the Williams–Beuren syndrome-deletion region at 7q11.23. Am J Hum Genet 2000;66:47–68.
66 Osborne LR, Li M, Pober B, Chitayat D, Bodurtha J, Mandel A, Costa T, Grebe T, Cox S, Tsui LC, Scherer SW: A 1.5 million-base pair inversion polymorphism in families with Williams-Beuren syndrome. Nat Genet 2001;29:321–325.
67 Lupski JR: Genomic disorders: recombination-based disease resulting from genome architecture. Am J Hum Genet 2003;72:246–252.
68 Gimelli G, Pujana MA, Patricelli MG, Russo S, Giardino D, Larizza L, Cheung J, Armengol L, Schinzel A, Estivill X, Zuffardi O: Genomic inversions of human chromosome 15q11-q13 in mothers of Angelman syndrome patients with class II (BP2/3) deletions. Hum Mol Genet 2003;12:849–858.
69 Gebhardt GS, Devriendt K, Thoelen R, Swillen A, Pijkels E, Fryns JP, Vermeesch JR, Gewillig M: No evidence for a parental inversion polymorphism predisposing to rearrangements at 22q11.2 in the DiGeorge/Velocardiofacial syndrome. Eur J Hum Genet 2003;11:109–111.
70 Saitta SC, Harris SE, Gaeth AP, Driscoll DA, McDonald-McGinn DM, Maisenbacher MK, Yersak JM, Chakraborty PK, Hacker AM, Zackai EH, Ashley T, Emanuel BS: Aberrant interchromosomal exchanges are the predominant cause of the 22q11.2 deletion. Hum Mol Genet 2004;13:417–428.
71 Shimokawa O, Kurosawa K, Ida T, Harada N, Kondoh T, Miyake N, Yoshiura K, Kishino T, Ohta T, Niikawa N, Matsumoto N: Molecular characterization of inv dup del(8p): analysis of five cases. Am J Med Genet 2004;128A:133–137.
72 Giglio S, Calvari V, Gregato G, Gimelli G, Camanini S, Giorda R, Ragusa A, Guerneri S, Selicorni A, Stumm M, Tonnies H, Ventura M, Zollino M, Neri G, Barber J, Wieczorek D, Rocchi M, Zuffardi O: Heterozygous submicroscopic inversions involving olfactory receptor-gene clusters mediate the recurrent t(4;8)(p16;p23) translocation. Am J Hum Genet 2002;71:276–285.
73 Jobling MA, Williams GA, Schiebel GA, Pandya GA, McElreavey GA, Salas GA, Rappold GA, Affara NA, Tyler-Smith C: A selective difference between human Y-chromosomal DNA haplotypes. Curr Biol 1998;8:1391–1394.
74 Repping S, Skaletsky H, Brown L, van Daalen SK, Korver CM, Pyntikova T, Kuroda-Kawaguchi T, de Vries JW, Oates RD, Silber S, van der Veen F, Page DC, Rozen S: Polymorphism for a 16-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. Nat Genet 2003;35:247–251.
75 Repping S, van Daalen SK, Korver CM, Brown LG, Marszalek JD, Gianotten J, Oates RD, Silber S, van der Veen F, Page DC, Rozen S: A family of human Y chromosomes has dispersed throughout northern Eurasia despite a 1.8-Mb deletion in the azoospermia factor c region. Genomics 2004;83:1046–1052.
76 Stefansson H, Helgason A, Thorleifsson G, Steinthorsdottir V, Masson G, Barnard J, Baker A, Jonasdottir A, Ingason A, Gudnadottir VG, Desnica N, Hicks A, Gylfason A, Gudbjartsson DF, Jonsdottir GM, Sainz J, Agnarsson K, Birgisdottir B, Ghosh S, Olafsdottir A, Cazier JB, Kristjansson K, Frigge ML, Thorgeirsson TE, Gulcher JR, Kong A, Stefansson K: A common inversion under selection in Europeans. Nat Genet 2005;37:129–137.
77 Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Maner S, Massa H, Walker M, Chi M, Navin N, Lucito R, Healy J, Hicks J, Ye K, Reiner A, Gilliam TC, Trask B, Patterson N, Zetterberg A, Wigler M: Large-scale copy number polymorphism in the human genome. Science 2004;305: 525–528.
78 Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, Scherer SW, Lee C: Detection of large-scale variation in the human genome. Nat Genet 2004;36:949–951.
79 Tuzun E, Sharp AJ, Bailey JA, Kaul R, Morrison VA, Pertz LM, Haugen E, Hayden H, Albertson D, Pinkel D, Olson MV, Eichler EE: Fine-scale structural variation of the human genome. Nat Genet 2005;37:727–732.
80 Sharp AJ, Locke DP, McGrath SD, Cheng Z, Bailey JA, Vallente RU, Pertz LM, Clark RA, Schwartz S, Segraves R, Oseroff VV, Albertson DG, Pinkel D, Eichler EE: Segmental duplications and copy-number variation in the human genome. Am J Hum Genet 2005;77:78–88.
81 Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Dohner H, Cremer T, Lichter P: Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances. Genes Chromosomes Cancer 1997;20:399–407.

82 Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG: High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 1998;20:207–211.
83 Yu W, Ballif BC, Kashork CD, Heilstedt HA, Howard LA, Cai WW, White LD, Liu W, Beaudet AL, Bejjani BA, Shaw CA, Shaffer LG: Development of a comparative genomic hybridization microarray and demonstration of its utility with 25 well-characterized 1p36 deletions. Hum Mol Genet 2003;12:2145–2152.
84 Locke DP, Segraves R, Nicholls RD, Schwartz S, Pinkel D, Albertson DG, Eichler EE: BAC microarray analysis of 15q11-q13 rearrangements and the impact of segmental duplications. J Med Genet 2004;41:175–182.
85 Shaw CJ, Shaw CA, Yu W, Stankiewicz P, White LW, Beaudet AL, Lupski JR: Comparative genomic hybridization using a proximal 17p BAC/PAC array detects rearrangements responsible for four genomic disorders. J Med Genet 2004;41:113–119.
86 Veltman JA, Yntema HG, Lugtenberg D, Arts H, Briault S, Huys EH, Osoegawa K, de Jong P, Brunner HG, Geurts van Kessel A, van Bokhoven H, Schoenmakers EF: High resolution profiling of X chromosomal aberrations by array comparative genomic hybridisation. J Med Genet 2004; 41:425–432.
87 Cai WW, Mao JH, Chow CW, Damani S, Balmain A, Bradley A: Genome-wide detection of chromosomal imbalances in tumors using BAC microarrays. Nat Biotechnol 2002;20:393–396.
88 Vissers LE, de Vries BB, Osoegawa K, Janssen IM, Feuth T, Choy CO, Straatman H, van der Vliet W, Huys EH, van Rijk A, Smeets D, van Ravenswaaij-Arts CM, Knoers NV, van der Burgt I, de Jong PJ, Brunner HG, van Kessel AG, Schoenmakers EF, Veltman JA: Array-based comparative genomic hybridization for the genomewide detection of submicroscopic chromosomal abnormalities. Am J Hum Genet 2003;73:1261–1270.
89 Shaw-Smith C, Redon R, Rickman L, Rio M, Willatt L, Fiegler H, Firth H, Sanlaville D, Winter R, Colleaux L, Bobrow M, Carter NP: Microarray based comparative genomic hybridisation (array-CGH) detects submicroscopic chromosomal deletions and duplications in patients with learning disability/mental retardation and dysmorphic features. J Med Genet 2004;41:241–248.
90 Ishkanian AS, Malloff CA, Watson SK, DeLeeuw RJ, Chi B, Coe BP, Snijders A, Albertson DG, Pinkel D, Marra MA, Ling V, MacAulay C, Lam WL: A tiling resolution DNA microarray with complete coverage of the human genome. Nat Genet 2004;36:299–303.
91 Bejjani BA, Saleki R, Ballif BC, Rorem EA, Sundin K, Theisen A, Kashork CD, Shaffer LG: Use of targeted array-based CGH for the clinical diagnosis of chromosomal imbalance: is less more? Am J Med Genet 2005;134A:259–267.
92 Cheung SW, Shaw CA, Yu W, Li JZ, Ou Z, Patel A, Yatsenko SA, Cooper ML, Furman P, Stankiewicz P, Lupski JR, Chinault AC, Beaudet AL: Development and validation of a CGH microarray for clinical cytogenetic diagnosis. Genet Med 2005;7:422–432.
93 Rickman L, Fiegler H, Shaw-Smith C, Nash R, Cirigliano V, Voglino G, Ling Ng B, Scott C, Whittaker J, Adinolfi M, Carter N, Bobrow M: Prenatal detection of unbalanced chromosomal rearrangements by array-CGH. J Med Genet 2005; doi: 10.1136/jmg.2005.037648.
94 Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO: Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet 1999;23:41–46.
95 Dhami P, Coffey AJ, Abbs S, Vermeesch JR, Dumanski JP, Woodward KJ, Andrews RM, Langford C, Vetrie D: Exon array-CGH: detection of copy number changes at the resolution of individual exons in the human genome. Am J Hum Genet 2005;76:750–762.
96 Lucito R, Healy J, Alexander J, Reiner A, Esposito D, Chi M, Rodgers L, Brady A, Sebat J, Troge J, West JA, Rostan S, Nguyen KC, Powers S, Ye KQ, Olshen A, Venkatraman E, Norton L, Wigler M: Representational oligonucleotide microarray analysis: a high-resolution method to detect genome copy number variation. Genome Res 2003;13:2291–2305.
97 Carvalho B, Ouwerkerk E, Meijer GA, Ylstra B: High resolution microarray comparative genomic hybridization analysis using spotted oligonucleotides. J Clin Pathol 2004;57:644–646.
98 Kriek M, White SJ, Bouma MC, Dauwerse HG, Hansson KB, Nijhuis JV, Bakker B, van Ommen GJ, den Dunnen JT, Breuning MH: Genomic imbalances in mental retardation. J Med Genet 2004;41:249–255.

99 Armour JAL, Sismani C, Patsalis PC, Cross G: Measurement of locus copy number by hybridisation with amplifiable probes. Nucleic Acids Res 2000;28:605–609.
100 Sismani C, Armour JA, Flint J, Girgalli C, Regan R, Patsalis PC: Screening for subtelomeric chromosome abnormalities in children with idiopathic mental retardation using multiprobe telomeric FISH and the new MAPH telomeric assay. Eur J Hum Genet 2001;9:527–532.
101 Hollox EJ, Atia T, Cross G, Parkin T, Armour JA: High throughput screening of human subtelomeric DNA for copy number changes using multiplex amplifiable probe hybridisation (MAPH). J Med Genet 2002;39:790–795.
102 Koolen DA, Nillesen WM, Versteeg MH, Merkx GF, Knoers NV, Kets M, Vermeer S, van Ravenswaaij CM, de Kovel CG, Brunner HG, Smeets D, de Vries BB, Sistermans EA: Screening for subtelomeric rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplification (MLPA). J Med Genet 2004;41:892–899.

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Gross Deletions and Translocations in Human Genetic Disease

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Abstract

Translocations and gross deletions constitute an important cause of both cancer and inherited disease. Such gene rearrangements are non-randomly distributed in the human genome as a consequence of selection for growth advantage and/or the inherent potential of some DNA sequences to be frequently involved in breakage and recombination. Chromosomal rearrangements are generated by a variety of recombinational processes, each characterised by mechanism-specific DNA sequence features. Various types of recombinogenic motifs have been shown to promote non-homologous end joining whilst direct repeats may mediate homologous recombination. In addition, repetitive sequence elements can facilitate the formation of secondary structure between DNA ends at translocation or gross deletion breakpoints, and in so doing, may play a role in illegitimate recombination. Although results from DNA breakpoint studies are broadly consistent with a role for homologous unequal recombination in deletion mutagenesis and a role for non-homologous recombination in the generation of translocations, homologous recombination and non-homologous end joining are unlikely to be mutually exclusive mechanisms. Thus, chromosomal rearrangements will often represent the net result of multiple highly complex molecular interactions that are not always readily explicable.

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Translocations and large deletions are an important cause of both cancer [1] and inherited disease [2]. Whilst deletions are associated with both inherited disease and cancer, translocations are almost exclusively somatic events that lead to neoplasia. Such chromosomal rearrangements are not located randomly but are rather confined to specific regions in the genome.

Non-Random Distribution of Gross Gene Rearrangements

For a variety of reasons, gross rearrangements are non-randomly distributed in the human genome [3]. Firstly, in order to come to clinical attention, somatic gene rearrangements associated with cancer must confer a growth advantage upon the affected cells or tissue, usually via gene deregulation or through the creation of a hybrid gene encoding a tumour-specific fusion protein [1]. By contrast, for germ-line gene rearrangements causing inherited disease to come to clinical attention, they must confer a disadvantage upon the individual, usually through haploinsufficiency.

Secondly, gross gene rearrangements are often associated with recombination 'hotspots', DNA sequences that promote either homologous unequal recombination or non-homologous recombination, the two main pathways of double strand break repair. Double strand breaks arise as a consequence of DNA damage but may also occur in programmed fashion during V(D)J recombination [4] or immunoglobulin heavy chain class switching [5]. Although the maintenance of genomic integrity requires the accurate repair of double strand breaks, genomic rearrangements may arise through their mis-repair.

Homologous Recombination

Homologous unequal recombination is typically mediated by very similar or identical non-allelic regions of homologous chromosomes, often containing interspersed repetitive elements such as *Alu* sequences or chromosome-specific low copy number repeats. This form of recombination between misaligned repeats on homologous chromosomes or sister chromatids serves to generate gross deletions and duplications [2]. However, if sequence exchange occurs between non-homologous chromosomes, such recombinational events may give rise to translocations. Deletions may also arise by homologous recombination mediated by similar or identical DNA regions within the same chromosome.

Non-Homologous Recombination

Non-homologous recombination occurs between sites that exhibit minimal sequence homology, yielding breakpoints that are characterized by microhomologies, short insertions and duplications as a consequence of staggered breaks or slipped mispairing followed by primed DNA synthesis [6]. Non-homologous end joining appears to require the presence of sequence signals, either highly specific (e.g. V(D)J recombination signal sequences) [4] or more

generic (e.g. immunoglobulin switch regions) [5], to ensure accurate somatic recombination. The presence of V(D)J or switch recombination-like sequences at both translocation [7] and deletion [8] breakpoint junctions has suggested that illegitimate V(D)J or class switch recombination events might underlie some chromosomal rearrangements. However, reports of further recombination- or cleavage-associated motifs such as the χ-like element [9], the translin binding site [10], alternating purine-pyrimidine tracts [11], curved or bent DNA [12] and recognition sites for topoisomerases I and II [13] at chromosomal rearrangement breakpoints hint at the involvement of other cellular processes. For many of these non-homologous signal sequences, the first reports suggesting their involvement with gross mutagenesis were based on a small number of motif observations at rearrangement breakpoint junctions. To determine whether such reports reflect bona fide associations or simply chance occurrence, the Gross Rearrangement Breakpoint Database (GRaBD) was established [3]. As we outline below, analysis of the considerable number of chromosomal breakpoint sequences in GRaBD (by comparison with a set of matched control DNA sequences) has revealed important new insights into the molecular mechanisms underlying gross gene rearrangements.

Multigene Study of Chromosomal Breakpoint Junctions

Initial analyses of the sequence context of chromosomal rearrangements were confined to the examination of small numbers of gross deletion and translocation breakpoint junctions at specific gene loci [3]. The construction of GRaBD, comprising some 397 chromosomal rearrangement breakpoint junctions [3], allowed us to extend these studies by facilitating the examination of the local DNA sequence environment of translocation and deletion breakpoints at a wide variety of different gene loci. This large multi-gene study of chromosomal breakpoints revealed the presence of: (i) DNA sequence features (over-represented at translocation breakpoints) associated with non-homologous recombination [3]; and (ii) repetitive sequence elements with the potential to form secondary structure intermediates between single-stranded DNA ends [14].

Oligonucleotide Composition of Translocation and Deletion Breakpoints

Measurement of oligonucleotide composition by comparing mononucleotide, dinucleotide and trinucleotide frequencies in the vicinity of translocation and deletion breakpoints with a set of reference DNA sequences using Fisher's exact test (table 1), revealed deletion and translocation breakpoint junction sequences to be

Table 1. Oligonucleotides significantly over-represented[a] at GRaBD deletion and translocation breakpoint junctions

Breakpoint type	Oligonucleotides	Oligonucleotide repeats	Tracts		Alternating purine-pyrimidine tracts
			polypurine	polypyrimidine	
Deletion breakpoints	A, T, TA, AT, AA, TAT, AAA, ATA, AAT, GTA, TAA, TAC, TGT, ATT, TTA, CAT	$(A)_{4–6}$, $(AT)_{2–4}$, $(TA)_{2–4}$, $(TG)_{3–8}$, $(GT)_{2–8}$, $(AC)_{2–4}$, $(CA)_3$, $(CCG)_{2–4}$, $(CGC)_{2–4}$, $(ACC)_3$, $(GCC)_{2–4}$, $(CTA)_2$, $(GTT)_2$	$(R)_{25–39}$	–	$(RY)_{1–36}$, $(YR)_{1–37}$
Translocation breakpoints	G, C, GG, CC, CG, GC, GGG, CGG, GGA, CCC, CCG, CCT, TGG, GGC, TCG, GAG, CGA, TCC	$(G)_{4–5}$, $(CA)_{3–8}$, $(AC)_{3–8}$, $(TAC)_{3–4}$, $(ACT)_2$, $(TGT)_3$, $(GTT)_3$	$(R)_{2–23}$	$(Y)_{2–44}$	–

[a]Significant at the 1% level.

AT-rich and GC-rich respectively (1% significance level). These findings run contrary to previous measurements of mononucleotide composition at such breakpoints. Some translocation breakpoints have been reported to be AT-rich [15], others GC-rich [16]. Regions that display a high level of recombination often appear to be GC-rich [17]. The over-representation of trinucleotides GGA, GGC, GGG, GAG, TGG and CCT in the vicinity of translocation breakpoints is however compatible with previous findings [18].

Formation of Non-B DNA Structures at Chromosomal Breakpoints

Specific DNA sequence motifs such as alternating purine-pyrimidine, polypurine and polypyrimidine tracts and G-rich tetrad direct repeats undergo structural transitions from the orthodox right-handed B-helical duplex to high energy state non-B DNA structures under negative superhelical stress. Non-B DNA conformations have been shown to coincide with chromosomal breakpoints [19]. These structures are thought to initiate genomic rearrangements by increasing the rate of formation of single-strand lesions at these sites.

Alternating purine-pyrimidine tracts of between 2 and 74 bp were found to be significantly over-represented in the vicinity of deletion breakpoint junctions (table 1). Such sequences, which have previously been noted to be associated with human recombination [20] and translocation [11] breakpoints, are prone to form Z-DNA particularly under conditions of negative superhelical stress [21]. Z-DNA is a left-handed helix with only a single minor groove that forms during transcription in vivo as a result of torsional strain generated by the moving RNA polymerase [21]. Z-DNA may be recombinogenic in a number of different ways. Firstly, it may facilitate recombination between homologous chromosomal regions by relieving the topological stress that arises when intact duplexes are intertwined [21]. Indeed, alternating purine-pyrimidine sequences are known to be preferentially cleaved by topoisomerase II [22]. Secondly, Z-DNA regions may exclude histones and other architectural proteins, thereby influencing both the location of nucleosomes and the organisation of chromosomal domains [21], as well as increasing accessibility to recombinases. Intriguingly in this context, sequences capable of Z-DNA formation have been shown to occur much more frequently in 5′ than in 3′ regions of human genes [21]. Finally, the presence of Z-DNA might render a region prone to illegitimate recombination by acting as a barrier to transcription [23]. During transcription, the DNA strands are unwound with one strand acting as a template for RNA chain elongation. Transcriptional arrest may thus serve to prolong the single-stranded state thereby making the DNA molecule more vulnerable to illegitimate recombination.

Polypurine runs of 2 to 23 bp and polypyrimidine tracts of 2 to 44 bp were significantly over-represented at translocation breakpoint junctions whereas polypurine tracts of 25 to 39 bp were over-represented at deletion breakpoint junctions (table 1). Such sequences have been previously reported to occur at both translocation [24] and gross deletion breakpoints [13] and appear to stimulate homologous recombination in vivo [25]. Polypurine and polypyrimidine sequences are prone to adopt the triple helical H-form of DNA particularly when exposed to an acidic environment and negative superhelical stress [26]. The triplex is formed when purine- or pyrimidine-rich single-stranded DNA, generated by the partial unwinding of the DNA helix, occupies the major groove of the intact portion of the double helix and forms Hoogsteen base-pairs with its purines [26]. Since H-DNA is partially single-stranded, it may be susceptible to nuclease attack that could then facilitate recombination. H-DNA may also promote recombination by blocking DNA replication. The ability of polypurine and polypyrimidine sequences to form H-DNA may thus render these sequences prone to illegitimate recombination.

Formation of DNA Secondary Structures between DNA Ends

Previous studies of gross rearrangement at specific gene loci [15, 18, 27] have served to document the occurrence of various types of repetitive sequence elements in the vicinity of breakpoint junctions. Inverted repeats (or palindromes) have been noted at breakpoint junctions [18] and when experimentally introduced into the mouse genome, such sequences are highly susceptible to rearrangement [27]. The analysis of the breakpoint junctions of some 77 Ewing sarcoma-related translocations [18] led to the proposal of an explanatory model of illegitimate recombination, based upon inter-strand hairpin loop formation, which provided the first indication that short repetitive sequences might be involved in non-homologous end joining through the formation of secondary structure between the DNA ends at translocation breakpoints. The potential involvement of local DNA sequence structure in double-strand break repair and inappropriate end joining was further examined by applying the technique of complexity analysis [14] to the GRaBD sequence data.

In DNA sequences, complexity is a measure of regularity, which may be calculated with respect to direct repeats, inverted repeats, inversions of inverted repeats or symmetric elements. A DNA sequence may be decomposed into a series of substrings (the number of which corresponds to the complexity score) with the length of each substring determined by the length of the longest matching repeat upstream of its position. The conditional complexity of a sequence may also be calculated by reference to another sequence with decomposition of

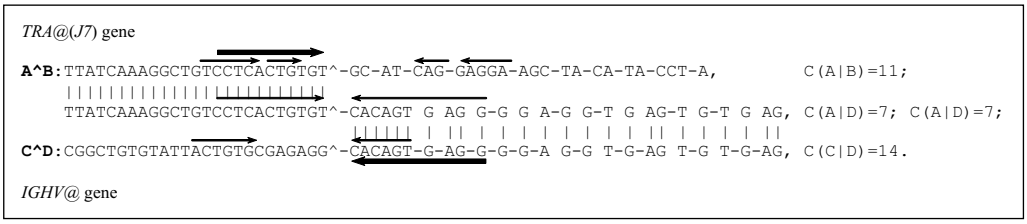
TRA@(J7) gene
A^B:TTATCAAAGGCTGTCCTCACTGTGT^-GC-AT-CAG-GAGGA-AGC-TA-CA-TA-CCT-A, C(A|B)=11;
TTATCAAAGGCTGTCCTCACTGTGT^-CACAGT G AG G-G G A-G G-T G AG-T G-T G AG, C(A|D)=7; C(A|D)=7;
C^D:CGGCTGTGTATTACTGTGCGAGAGG^-CACAGT-G-AG-G-G-G-A G-G T-G-AG T-G T-G-AG, C(C|D)=14.
IGHV@ gene

Fig. 1. Inversion/translocation in the *TRA@*(*J7*) and *IGHV* genes which could have been mediated by an inverted repeat.

the former being based on the repeats within the latter. In this manner, conditional complexity was measured for 5′ and 3′ breakpoint-flanking sequences ('ends') in both wild-type and mutant alleles and was used to assess the potential of these flanking sequences to form secondary structure intermediates [14]. To drive the rearrangement process, it is assumed that such secondary structures should be both more prominent and more stable than those associated with the original sequences. For example, an inverted repeat was found to be able to account for the inversion/translocation that occurred between the *TRA@*(*J7*) (MIM#186880) and *IGHV@*(MIM#147070) genes (GRaBD ID Nos 77 and 78). Figure 1 illustrates the appearance of an inverted repeat between the 5′-end of the first sequence and the 3′-end of the second sequence that is more prominent than those existing in the original sequences.

GRaBD deletion and translocation breakpoint sequences were found to be mediated by direct repeats, inverted repeats, symmetric elements and inversions of inverted repeats. Thirteen cases were inferred to be mediated by homologous recombination on the grounds that direct repeats were present at the paired breakpoints. All appear only to be involved in mediating simple deletions. Seven of the 12 direct repeats mediating deletions corresponded to *Alu* sequences.

Alu Sequences as Mediators of Deletions and Translocations

All sequences in the GRaBD database were screened for the presence of repetitive elements using the Repbase database (http://www.girinst.org) and the RepeatMasker program (http://woody.embl-heidelberg.de/repeatmask). A total of 102 repetitive sequence elements were noted in 93 different GRaBD sequences, but only 80 of these elements spanned the breakpoint junctions (table 2). The *Alu* sequence element was found to be the most common interspersed repeat at GRaBD breakpoints.

Table 2. Repetitive sequences spanning GRaBD deletion and translocation breakpoints

Type of repetitive element		$N_{elements}$	N^{a}_{both}	N^{b}_{one}
SINEs	*Alu*	47	7	29
	MIR	3	0	3
LINEs	LINE1	21	0	17
	LINE2	2	0	1
LTR	MaLR	2	0	1
	ERVK	1	0	0
DNA	MER1-type	2	0	1
	MER2-type	2	0	0

[a]Number of breakpoint sequence pairs in which repetitive elements occur in both sequences.

[b]Number of breakpoint sequence pairs in which repetitive elements occur in one of the sequences.

Alu sequence-mediated homologous recombination is a frequent cause of both genetic disease and cancer, whether in the germ-line generating gene deletions [13, 28], or in the soma mediating translocations [30]. In the GRaBD database, *Alu* sequences were found to span both breakpoints in seven cases of gross deletion that may therefore be inferred to have arisen by homologous recombination [3]. *Alu* sequences found in the vicinity of single breakpoints could however still have mediated the corresponding rearrangements by non-homologous recombination since a core 26-bp sequence within the *Alu* repeat is reportedly recombinogenic [30].

Non-Homologous Recombination Signals at Translocation Breakpoints

The frequency of occurrence of sequence motifs directly or indirectly associated with a variety of mechanisms of non-homologous recombination (table 3) was measured within regions ± 15 bp flanking the breakpoints [3]. To determine whether these motifs were disproportionately located at particular positions, their frequency of occurrence was also measured at each position individually within the range ± 10 bp of the breakpoints and was compared to their respective frequency in the reference DNA set using Fisher's exact test. A total of 16 of the 37 recombination-associated motifs tested (table 3) were

Table 3. DNA sequence motifs known to be associated with site-specific recombination, mutation, cleavage and gene rearrangement

Motif description[a]	Motif sequence(s)[b]
Vertebrate/plant topoisomerase I consensus cleavage sites	CAT, CTY, GTY, RAT
Vaccinia topoisomerase I consensus cleavage site	YCCTT
Vertebrate topoisomerase II consensus cleavage site	RNYNNCNNGYNGKTNYNY
Drosophila topoisomerase II consensus cleavage site	GTNWAYATTNATNNR
Heptamer recombination signal	CACAGTG
Nonamer recombination signal	ACAAAAACC
Immunoglobulin heavy chain class switch repeats	GAGCT, GGGCT, GGGGT, TGGGG, TGAGC
Translin target sites	ATGCAG, GCCCWSSW
χ-element	GCTGGTGG
Human hypervariable minisatellite core sequence	GGGCAGGANG
Human minisatellite conserved sequence/ χ-like element	GCWGGWGG
Human hypervariable minisatellite core sequence	GGAGGTGGGCAGGARG
Human hypervariable minisatellite recombination sequence	AGAGGTGGGCAGGTGG
Mariner transposon-like element (3′ end)	GAAAATGAAGCTATTTACCCAGGA
Deletion hotspot consensus sequence	TGRRKM
Murine MHC deletion hotspot	$(CAGR)_n$
Murine parvovirus recombination hotspot	CTWTTY
Murine MHC recombination hotspot	CAGRCAGR
Murine LTR recombination hotspot	TGGAAATCC
DNA polymerase α pause site core sequence	GAG, ACG, GCS
DNA polymerase arrest site	WGGAG
DNA polymerase α frameshift hotspots	TCCCCC, CTGGCG
DNA polymerase β frameshift hotspots	ACCCWR, TTTT
DNA polymerase α/β frameshift hotspots	TGGNGT, ACCCCA

[a]See Abeysinghe et al. [3] for a complete list of references.

[b]The ambiguity code symbols are as follows: R = A/G, Y = C/T, K = G/T, M = A/C, S = G/C, W = A/T, N = A/C/G/T.

Table 4. Motifs significantly over-represented within ±15 bp of GRaBD translocation breakpoints

Motif type	Motif	Observed[a]	Expected[b]	*P* value
Heptamer recombination signal	CACAGTG	10	1.4	2.8×10^{-6}
DNA polymerase α pause site core sequence	GCS	164	132.8	4.8×10^{-3}
DNA polymerase α frameshift hotspot	TCCCCC	5	1.7	3.1×10^{-2}
DNA polymerase α pause site core sequence	GAG	139	118.1	3.2×10^{-2}

[a]Frequency of occurrence within GRaBD translocation breakpoints.
[b]Average frequency of occurrence within a matched reference control sequence sample.

found to be significantly over-represented either within the ±15-bp flanking sequence (table 4) or at specific positions relative to the translocation breakpoints (table 5). However, with the exception of the translin binding site complement CTGCAT [10] at positions −8 ($P = 0.02$) and +8 ($P = 0.02$), and the immunoglobulin switch repeat TGAGC [29] which was significantly over-represented within the ±15-bp flanking regions ($P = 0.05$), none of the motifs listed in table 3 were significantly over-represented in close proximity to the deletion breakpoints.

V(D)J Recombination-Mediated Chromosomal Rearrangement

The V(D)J recombination signal sequence comprises two recombination signal sequences of 7 bp (heptamer) and 9 bp (nonamer) separated by a non-conserved spacer sequence of 12 ± 1 or 23 ± 1 nucleotides [4]. It serves to mediate the cutting and rejoining of all variable (V), diversity (D) and joining (J) coding segments of immunoglobulin and T cell receptor genes [4]. The lymphoid-specific process of V(D)J recombination is initiated by RAG1 binding to the nonamer and then catalysing double-strand breaks precisely at the borders of the heptamers and the adjacent coding regions [4]. Subsequent ligation of a pair of gene sequences occurs only if they are flanked by recombination signal sequences of different spacer lengths (‘the 12/23 rule’).

The heptamer motif was significantly over-represented ($P < 0.05$) at specific positions overlapping (−3), and immediately adjacent to (−7), the

Table 5. Significantly over-represented motifs at specific positions relative to GRaBD translocation breakpoints

Motifs overlapping or immediately flanking breakpoints[a]	Motif	Position	*P* value
Motif type			
DNA polymerase α pause site core sequence	ACG	−1	3.11E-03
Immunoglobulin heavy chain class switch repeat[b]	GCTCA	−2	9.22E-03
Fragile X breakpoint cluster repeat	CGG	+1	9.24E-03
Immunoglobulin heavy chain class switch repeat[b]	CCCCA	−3	1.37E-02
Heptamer recombination signal[b]	CACTGTG	−7	1.65E-02
Heptamer recombination signal[b]	CACTGTG	−3	1.89E-02
Heptamer recombination signal	CACAGTG	−7	2.42E-02
Human minisatellite conserved sequence/χ-like element	GCWGGWGG	−1	2.75E-02
DNA polymerase α/β frameshift hotspot	TGGGGT	−5	3.39E-02
Motifs at positions within 10 bp of breakpoints			
Fragile X breakpoint cluster repeat	CGG	−10	1.01E-02
DNA polymerase α frameshift hotspot[b]	GGGGGA	+2	1.73E-02
DNA polymerase arrest site[b]	CTCCW	−9	2.16E-02
Translin target site	ATGCAG	+5	2.66E-02
Translin target site	GCCCWSSW	−10	2.77E-02
DNA polymerase α pause site core sequence	ACG	+8	3.22E-02
Translin target site	GCCCWSSW	−9	3.75E-02
Murine MHC deletion hotspot[b]	YCTG	−7	4.79E-02

[a]See Abeysinghe et al. [3] for a complete list of references.
[b]Complement of motif.

translocation breakpoints (table 5). Although the complement of the nonamer element was not significantly over-represented in the vicinity of the translocation breakpoints, it was found to be over-represented ($P < 0.01$) at positions −40 and −35 [3]. These positions were precisely 24 and 23 nucleotides away from the heptamer motifs at positions −7 and −3 respectively. The relative positions of these motifs, together with the juxtaposition of the heptamer to the breakpoints, are consistent with the view that the process of V(D)J recombination could have been responsible for at least some of the GRaBD translocations through the illegitimate use of recombination signal-like sequences. Recombination signal-like sequences have previously been reported at a number of different translocation breakpoints [7] and such illegitimate sites have been shown to be capable of acting as substrates for V(D)J recombination in vitro [31].

V(D)J recombination serves to fuse the antigen receptor locus to another unlinked locus by virtue of its possession of a cryptic recombination signal-like sequence.

In total, there were ten occurrences of the heptamer motif or its complement within the ±15-bp flanking sequence of translocation breakpoints (table 4). This signified a heavy over-representation when compared to its frequency in the matched controls ($P < 0.001$). Seven of the ten occurrences of the heptamer within the ±15-bp flanking region were located within immunoglobulin and T-cell receptor gene loci. Such sites, if involved in V(D)J recombination-mediated rearrangement, would represent the illegitimate use of legitimate recombination sites (in contrast to sites used at non-antigen receptor loci which would constitute the illegitimate use of illegitimate sites).

Chromosomal Rearrangement and Replication Slippage

Replication slippage is a form of unequal recombination between short direct repeats that occurs at stalled replication forks [32]. Replication fork arrest is initiated by DNA polymerase pausing, triggered by the presence of e.g. hairpin loop-forming inverted repeats or polypurine stretches, which leads to the dissociation of the polymerase from the DNA molecule [33]. Upon dissociation, the newly synthesised strand separates from the template strand at one copy of a direct repeat and subsequently re-anneals at the other. Reinitiation of the replication fork then commences from the newly aligned site [33]. This model of replication slippage predicts that both the intervening sequence between the direct repeats and the direct repeat at the site of initial disassociation should loop out and be excised in the ensuing repair process [32]. Replication arrest actually stimulates strand slippage and can trigger both homologous and non-homologous recombination leading to DNA rearrangement [32].

Six motifs associated with replication slippage were found to be significantly over-represented, either at specific positions or more generally flanking GRaBD translocation breakpoints [3]. Three of these (ACG, GCS and GAG) are triplets at which DNA polymerase α has a propensity to stall in the presence of inverted repeats [34]. Another two were TCCCCC, significantly over-represented in the general vicinity of translocation breakpoints at the 5% level of significance (table 4), and TGGGGT, significantly over-represented ($P < 0.05$) at position −5, overlapping the translocation breakpoints (table 5). These are known frameshift mutation hotspots for rat α and/or β DNA polymerase in vitro [35, 36]. Finally, the triplet CGG, significantly over-represented immediately adjacent to the GRaBD translocation breakpoints at position +1, is also known to be associated with replication slippage in vivo. The CGG triplet

repeat in the *FMR1* gene (OMIM: 309550) is expanded in fragile X mental retardation syndrome through a mechanism which probably involves replication slippage between Okazaki fragments [37]. Interestingly, CCG (the complement of CGG) repeats are also known to be associated with deletional mutagenesis [38], a finding borne out by our own results (table 1).

Immunoglobulin Heavy Chain Class Switch Recombination-Mediated Chromosomal Rearrangement

Immunoglobulin class switch repeats occur upstream of all constant region genes in a tandem arrangement of 1 to 10 kb and consist of tandem arrays of G-rich pentanucleotide repeats, GAGCT, GGGCT or GGGGT that serve to mediate heavy chain class switch recombination [29]. Such a motif (TGAGC) was found to be significantly over-represented ($P < 0.05$) within the $\pm$15-bp sequences flanking GRaBD deletion breakpoints; whilst complements of these repeats were identified at positions -2 (GCTCA, $P < 0.01$) and -3 (CCCCA, $P < 0.05$) overlapping the GRaBD translocation breakpoints [3]. Immunoglobulin heavy chain class switch recombination is a process of non-homologous recombination that serves to replace DNA sequences encoding the constant region of an immunoglobulin heavy chain gene by sequences from a constant region gene further downstream. Switch repeats have been noted at translocation breakpoints associated with human haematological malignancies and, to a lesser degree, have also been reported at deletion breakpoints. The presence of switch region motifs in the vicinity of deletion and translocation breakpoints is consistent with the view that illegitimate class switch recombination constitutes an important mechanism of chromosomal rearrangement. It should be noted, however, that the primary sequences of switch regions are not critical for class switch recombination. Rather, it is the secondary structures formed by tandem pentameric repeats that act as signals to mediate the process [39].

A Role for χ Elements in Chromosomal Rearrangement?

The χ element, a mediator of prokaryotic recombination through stimulation of RecBCD-dependent recombination in its vicinity [40], was found to be significantly over-represented ($P < 0.05$) overlapping the GRaBD translocation breakpoints at position -1 [3]. Conserved χ-like elements are present in human minisatellites [9] and also within the recombinogenic 26-bp *Alu* sequence core [30]. χ-like elements have previously been reported in association not only with

oncogenic translocation breakpoints [9] but also with the breakpoints of gross deletions causing inherited disease [41]. It has been suggested that χ-like sequence elements may represent a distinct class of recognition element for V(D)J recombinase [7].

Translin-Mediated Chromosomal Rearrangement

Translin is a multimeric protein that recognises the single-stranded ends of staggered breaks as a first step to DNA repair [42]. Translin target sequences occurring at translocation breakpoints not associated with V(D)J recombinases were first described in lymphoid neoplasms [10]. Potential translin binding sites have since been found at translocation breakpoints in both solid tumours [43] and haematological malignancies [10]. Consensus translin binding sites were found to be significantly over-represented ($P < 0.05$) at positions +5 (ATGCAG), −9 and −10 (GCCCWSSW) relative to the translocation breakpoints [3]. The complement of one of these motifs, CTGCAT, was found to be over-represented ($P = 0.02$) at positions −8 and +8 relative to deletion breakpoints [3].

Topoisomerase Cleavage Sites and Chromosomal Rearrangement

Topoisomerase consensus II cleavage sites have been observed in the vicinity of translocation breakpoints at a variety of genes underlying both solid [44] and haematological neoplasms [45]. To a lesser degree, such sites have been reported at inherited disease-associated deletion breakpoints [46]. Also, topoisomerase I sites have been observed at translocation [47] and deletion [13] breakpoints.

Despite a plethora of previous reports of the presence of both topoisomerase I [13, 47] and topoisomerase II [44, 45] cleavage sites at specific translocation breakpoints, cleavage sites for these unwinding enzymes were not found to be significantly over-represented at GRaBD breakpoint junctions [3]. Since topoisomerase cleavage sites are either short or highly redundant or both, their chance occurrence at breakpoint junctions is unlikely to be infrequent. Clearly, their presence at a given translocation or deletion breakpoint should not be automatically taken to imply that they are directly involved in the process of rearrangement.

Concluding Remarks

Although results from the GRaBD analysis broadly support a homologous recombination-based model of deletion mutagenesis, they reveal a paucity of non-homologous recombination DNA signals at deletion breakpoints. This is somewhat surprising in view of the fact that gross gene deletions, like translocations, have been reported in association with a variety of such sequences, including topoisomerase consensus sequences [13, 46], V(D)J recombination signal-like sequences [8] and χ-like elements [41]. This would suggest that many of these reports simply reflect the chance occurrence of motifs in the vicinity of deletion breakpoint(s) rather than bona fide associations [3]. By contrast, the results of the GRaBD analysis are consistent with the notion that translocations may be generated by non-homologous recombination occurring between sites of minimal sequence homology with both legitimate and illegitimate recombination signals.

In reality, the presence of specific recombinogenic motifs is not obligatory and different recombination signals may act in concert. Thus, in addition to their ability to mediate homologous recombination, *Alu* sequences contain a 26-bp core sequence with homology to χ [30] and sequences homologous to translin-binding sequences both of which may serve to promote non-homologous recombination. χ-like sequence elements, hairpin loops (inverted repeats) and single strand-double strand DNA junctions may all serve as illegitimate recognition elements for the V(D)J recombinase [7].

This chapter has attempted to summarize what is now actually quite a large body of data on the various DNA sequences shown to be over-represented in the vicinity of gross deletions and translocations associated with human genetic disease. Although a variety of different types of sequence, including repetitive elements and recombinogenic motifs, appear to be involved, it is still unclear precisely how these sequences come together to promote deletion/translocation mutagenesis. One possibility is that these sequences have a common ability to facilitate or promote secondary structure formation, particularly the adoption of non-B DNA structures. These abnormal structures may then interfere with the normal cellular processes of DNA replication, transcription, and conceivably also DNA repair and in so doing, promote inappropriate recombination between pre-existing micro-homologies.

A variety of other factors may also be involved in promoting chromosomal rearrangement such as the physical proximity of certain genes on non-homologous chromosomes during specific phases of the cell cycle as a consequence of the precise spatial positioning of chromosomes [48]. This notwithstanding, it may well be that the variety of recombinogenic sequence motifs, combined with the very considerable length of the sequences involved, will serve to ensure that the

complexity of the underlying mutational mechanisms will often continue to defy simple explanation.

References

1 Rabbitts TH: Chromosomal translocations in human cancer. Nature 1994;372:143–149.
2 Stankiewicz P, Lupski JR: Genome architecture, rearrangements and genomic disorders. Trends Genet 2002;18:74–82.
3 Abeysinghe SS, Chuzhanova N, Krawczak M, Ball EV, Cooper DN: Translocations and gross deletion breakpoints in human inherited disease and cancer I: nucleotide composition and recombination-associated motifs. Hum Mutat 2003;22:229–244.
4 Bassing CH, Swat W, Alt FW: The mechanism and regulation of chromosomal V(D)J recombination. Cell 2002;109(suppl):S45–S55.
5 Zhang K: Immunoglobulin class switch recombination machinery: progress and challenges. Clin Immunol 2000;95:1–8.
6 Sargent RG, Brenneman MA, Wilson JH: Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. Mol Cell Biol 1997;17: 267–277.
7 Davila M, Foster S, Kelsoe G, Yang K: A role for secondary V(D)J recombination in oncogenic chromosomal translocations? Adv Cancer Res 2001;81:61–92.
8 Karsten SL, Lagerstedt K, Carlberg BM, Kleijer WJ, Zaremba J, Van Diggelen OP, Czartoryska B, Pettersson U, Bondeson ML: Two distinct deletions in the IDS gene and the gene W: a novel type of mutation associated with the Hunter syndrome. Genomics 1997;43:123–129.
9 Krowczynska AM, Rudders RA, Krontiris TG: The human minisatellite consensus at breakpoints of oncogene translocations. Nucleic Acids Res 1990;18:1121–1127.
10 Aoki K, Suzuki K, Sugano T, Tasaka T, Nakahara K, Kuge O, Omori A, Kasai M: A novel gene, translin, encodes a recombination hotspot binding protein associated with chromosomal translocations. Nat Genet 1995;10:167–174.
11 Thandla SP, Ploski JE, Raza-Egilmez SZ, Chhalliyil PP, Block AW, deJong PJ, Aplan PD: ETV6-AML1 translocation breakpoints cluster near a purine/pyrimidine repeat region in the ETV6 gene. Blood 1999;93:293–299.
12 Nobile C, Toffolatti L, Rizzi F, Simionati B, Nigro V, Cardazzo B, Patarnello T, Valle G, Danieli GA: Analysis of 22 deletion breakpoints in dystrophin intron 49. Hum Genet 2002;110:418–421.
13 Cao X, Eu KW, Seow-Choen F, Zhao Y, Cheah PY: Topoisomerase-I- and Alu-mediated genomic deletions of the APC gene in familial adenomatous polyposis. Hum Genet 2001;108:436–442.
14 Chuzhanova N, Abeysinghe SS, Krawczak M, Cooper DN: Translocations and gross deletion breakpoints in human inherited disease and cancer II: potential involvement of repetitive sequence elements in secondary structure formation between DNA ends. Hum Mutat 2003;22: 245–251.
15 Edelmann L, Spiteri E, Koren K, Pulijaal V, Bialer MG, Shanske A, Goldberg R, Morrow BE: AT-rich palindromes mediate the constitutional t(11;22) translocation. Am J Hum Genet 2001;68: 1–13.
16 Cohen O, Cans C, Cuillel M, Gilardi JL, Roth H, Mermet MA, Jalbert P, Demongeot J: Cartographic study: breakpoints in 1574 families carrying human reciprocal translocations. Hum Genet 1996;97:659–667.
17 Yu A, Zhao C, Fan Y, Jang W, Mungall AJ, Deloukas P, Olsen A, Doggett NA, Ghebranious N, Broman KW, Weber JL: Comparison of human genetic and sequence-based physical maps. Nature 2001;409:951–953.
18 Zucman-Rossi J, Legoix P, Victor J-M, Lopez B, Thomas G: Chromosome translocation based on illegitimate recombination in human tumors. Proc Natl Acad Sci USA 1998;95:11786–11791.
19 Bacolla A, Jaworski A, Larson JE, Jakupciak JP, Chuzhanova N, Abeysinghe SS, O'Connell CD, Cooper DN, Wells, RD: Breakpoints of gross deletions coincide with non-B DNA conformations. Proc Natl Acad Sci USA 2004;101:14162–14167.

20 Wahls WP, Wallace LJ, Moore PD: Hypervariable minisatellite DNA is a hotspot for homologous recombination in human cells. Cell 1990;60:95–103.
21 Herbert A, Rich A: Left-handed Z-DNA: structure and function. Genetica 1999;106:37–47.
22 Spitzner JR, Chung IK, Muller MT: Eukaryotic topoisomerase II preferentially cleaves alternating purine-pyrimidine repeats. Nucleic Acids Res 1990;18:1–11.
23 Peck LJ, Wang JC: Transcriptional block caused by a negative supercoiling induced structural change in an alternating CG sequence. Cell 1985;40:129–137.
24 Hirai H, Ogawa S, Kurokawa M, Yazaki Y, Mitani K: Molecular characterization of the genomic breakpoints in a case of t(3;21)(q26;q22). Genes Chromosomes Cancer 1999;26:92–96.
25 Rooney SM, Moore PD: Antiparallel, intramolecular triplex DNA stimulates homologous recombination in human cells. Proc Natl Acad Sci USA 1995;92:2141–2144.
26 Frank-Kamenetskii MD, Mirkin SM: Triplex DNA structures. Annu Rev Biochem 1995;64:65–95.
27 Akgün E, Zahn J, Baumes S, Brown G, Liang F, Romanienko PJ, Lewis S, Jasin M: Palindrome resolution and recombination in the mammalian germ line. Mol Cell Biol 1997;17:5559–5570.
28 Harteveld KL, Losekoot M, Fodde R, Giordano PC, Bernini LF: The involvement of Alu repeats in recombination events at the α-globin gene cluster: characterization of two α^0-thalassaemia deletion breakpoints. Hum Genet 1997;99:528–534.
29 Ohno S: (AGCTG) (AGCTG) (AGCTG) (GGGTG) as the primordial sequence of intergenic spacers: the role in immunoglobulin class switch. Differentiation 1981;18:65–74.
30 Rüdiger NS, Gregersen N, Kielland-Brandt MC: One short well conserved region of Alu-sequences is involved in human gene rearrangements and has homology with prokaryotic chi. Nucleic Acids Res 1995;23:256–260.
31 Raghavan SC, Kirsch IR, Lieber MR: Analysis of the V(D)J recombination efficiency at lymphoid chromosomal translocation breakpoints. J Biol Chem 2001;276:29126–29133.
32 Michel B: Replication fork arrest and DNA recombination. Trends Biochem Sci 2000;25: 173–178.
33 Viguera E, Canceill D, Ehrlich SD: Replication slippage involves DNA polymerase pausing and dissociation. EMBO J 2001;20:2587–2595.
34 Weaver DT, DePamphilis ML: Specific sequences in native DNA that arrest synthesis by DNA polymerase alpha. J Biol Chem 1982;257:2075–2086.
35 Kunkel TA: The mutational specificity of DNA polymerase-beta during in vitro DNA synthesis. Production of frameshift, base substitution, and deletion mutations. J Biol Chem 1985;260: 5787–5796.
36 Kunkel TA: The mutational specificity of DNA polymerases-alpha and -gamma during in vitro DNA synthesis. J Biol Chem 1985;260:12866–12874.
37 Jin P, Warren ST: Understanding the molecular basis of fragile X syndrome. Hum Mol Genet 2000;9:901–908.
38 Jones C, Mullenbach R, Grossfeld P, Auer R, Favier R, Chien K, James M, Tunnacliffe A, Cotter F: Co-localisation of CCG repeats and chromosome deletion breakpoints in Jacobsen syndrome: evidence for a common mechanism of chromosome breakage. Hum Mol Genet 2000;9: 1201–1208.
39 Tashiro J, Kinoshita K, Honjo T: Palindromic but not G-rich sequences are targets of class switch recombination. Int Immunol 2001;13:495–505.
40 Kowalczykowski SC, Dixon DA, Eggleston AK, Lauder SD, Rehrauer WM: Biochemistry of homologous recombination in *Escherichia coli*. Microbiol Rev 1994;58:401–465.
41 Lopez-Correa C, Dorschner M, Brems H, Lazaro C, Clementi M, Upadhyaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns JP, Marynen P, Stephens K, Legius E: Recombination hotspot in *NF1* microdeletion patients. Hum Mol Genet 2001;10:1387–1392.
42 Kasai M, Matsuzaki T, Katayanagi K, Omori A, Maziarz RT, Strominger JL, Aoki K, Suzuki K: The translin ring specifically recognizes DNA ends at recombination hot spots in the human genome. J Biol Chem 1997;272:11402–11407.
43 Hosaka T, Kanoe H, Nakayama T, Murakami H, Yamamoto H, Nakamata T, Tsuboyama T, Oka M, Kasai M, Sasaki MS, Nakamura T, Toguchida J: Translin binds to the sequences adjacent to the breakpoints in the TLS and CHOP genes in liposarcomas with translocation t(12;6). Oncogene 2000;19:5821–5825.

44 Obata K, Hiraga H, Nojima T, Yoshida MC, Abe S: Molecular characterization of the genomic breakpoint junction in a t(11;22) translocation in Ewing sarcoma. Genes Chromosomes Cancer 1999;25:6–15.
45 Mercher T, Busson-Le Coniat M, Khac FN, Ballerini P, Mauchauffe M, Bui H, Pellegrino B, Radford I, Valensi F, Mugneret F, Dastugue N, Bernard OA, Berger R: Recurrence of OTT-MAL fusion in t(1;22) of infant AML-M7. Genes Chromosomes Cancer 2002;33:22–28.
46 Otto E, Betz R, Rensing C, Schätzle S, Kuntzen T, Vetsi T, Imm A, Hildebrandt F: A deletion distinct from the classical recombination of juvenile nephronophthisis type 1 (NPH1) allows exact molecular definition of deletion breakpoints. Hum Mutat 2000;16:211–223.
47 Tashiro S, Kotomura N, Tanaka K, Suzuki K, Kyo T, Dohy H, Niwa O, Kamada N: Identification of illegitimate recombination hot spot of the retinoic acid receptor alpha gene involved in 15;17 chromosomal translocation of acute promyelocytic leukaemia. Oncogene 1994;9:1939–1945.
48 Kozubek S, Lukásová E, Jirsová P, Koutna I, Kozubek M, Ganova A, Bartova E, Falk M, Pasekova R: 3D structure of the human genome: order in randomness. Chromosoma 2002;111:321–331.

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Nucleotide Excision Repair and Related Human Diseases

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Abstract

Nucleotide excision repair (NER) of DNA-lesions is the most versatile DNA repair mechanism involved in genome maintenance, cell and organismal preservation. Deciphering the stepwise mechanism of NER has mostly relied on cells from rare patients presenting photosensitive, recessively inherited genetic disorders such as xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne (CS) syndromes. Cells from these patients share various extents of impaired capacity of repairing UV-induced DNA lesions (cyclobutane pyrimidine dimers, 6-4 pyrimidine-pyrimidone photo products) located either in transcribed DNA strands or in inactive DNA. We review here the essentials of NER actors and how impairment of their activity may lead to distinct and characteristic human disorders whose presentation may be limited to developmental trait (TTD; CS), or cumulate with cancer susceptibility toward genotoxic aggressions, most notably short wavelength ultraviolets.

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Content and structure of the DNA molecule are continuously compromised either upon exposure to lesion-inducing agents such as chemicals and radiations or by endogenously produced oxidative metabolites. The presence of DNA lesions compromises cellular functions such as DNA replication and transcription. Overloading of repair capacity of a cell may ultimately result in programmed cell death. Otherwise, replication of DNA lesions may be mutagenic and yield instability at nucleotide and chromosome levels. Carcinogenesis is the major outcome of DNA mutagenesis.

In order to prevent the detrimental consequences of DNA alterations, cells from bacteria to superior eukaryotes have evolved several lines of protections, among which, DNA repair mechanisms adapted for the removal of the different classes of damages is probably one of the first line of protection. Nucleotide excision repair (NER) is the most versatile DNA repair pathway devoted to recognition

and removal of a wide range of structurally unrelated DNA lesions. Ultraviolet wavelengths contained in the solar light (290–700 nm) constitute a major genotoxic stress. UVB (290–320 nm) are absorbed in the cellular DNA to introduce the prototype UV photoproducts, CPD (cis-syn-cyclobutane dimers) and 6-4PP (pyrimidine (6-4) pyrimidone), both of which are major substrates of NER.

NER relies on the contribution of about 30 polypeptides involved in a stepwise process as follows: 1) recognition of the DNA lesion; 2) formation and stabilization of a multiprotein complex containing TFIIH; 3) single strand incision at both sides of the lesion; 4) excision of the lesion-containing single stranded DNA fragment; 5) DNA repair synthesis to replace the excised oligo nucleotides; 6) 5′ and 3′ ligation of the newly synthesized DNA strand. In the mid-1980s, Philip Hanawalt and colleagues observed faster NER in actively transcribed genes than in transcriptionally silent regions of mammalian genomes [1]. On the basis of this important result, the existence of two NER subpathways was then demonstrated. One NER subpathway, coupled to transcription is called Transcription Coupled Repair (TCR). TCR preferentially removes DNA damages from the transcribed strand of active genes. The other NER subpathway is called Global Genome Repair (GGR). GGR removes lesions throughout the genome including those in the non-transcribed strand of active genes.

Xeroderma pigmentosum, trichothiodystrophy and Cockayne syndrome are inborn recessive NER deficiencies associated with photosensitivity and characteristic clinical features. Seven complementation groups have been determined for the XP syndrome (XP-A to XP-G), five for CS (CS-A and CS-B, XP-B/CS, XP-D/CS, XP-G/CS) and three for photosensitive TTD (XPB, XPD and TTD-A). Mutations in the *XPB*, *XPD* and *XPG* genes may thus lead to either one of these distinct syndromes. Use of cells from these patients has greatly contributed to deciphering the stepwise NER pathway and to establish genotype-phenotype correlations.

The Nucleotide Excision Repair Pathway

The order of assembly of NER factors has been the subject of debate. Although it has been proposed that the repair machinery could be recruited as a preformed repairosome complex [2] or as part of the RNA pol II holoenzyme [3], a more unifying model currently relies on the sequential assembly of NER factors at the site of DNA damage [4].

Recognition of DNA Lesion

Damage recognition not only is the first but also probably the most delicate step of NER. It has first to determine whether a DNA distortion corresponds to

a bona fide substrate of NER and second to take into account the specific chromatin context [5]. This indicates how modular must be the appropriate sets of proteins required for the recognition of specific DNA damages by either GGR or TCR.

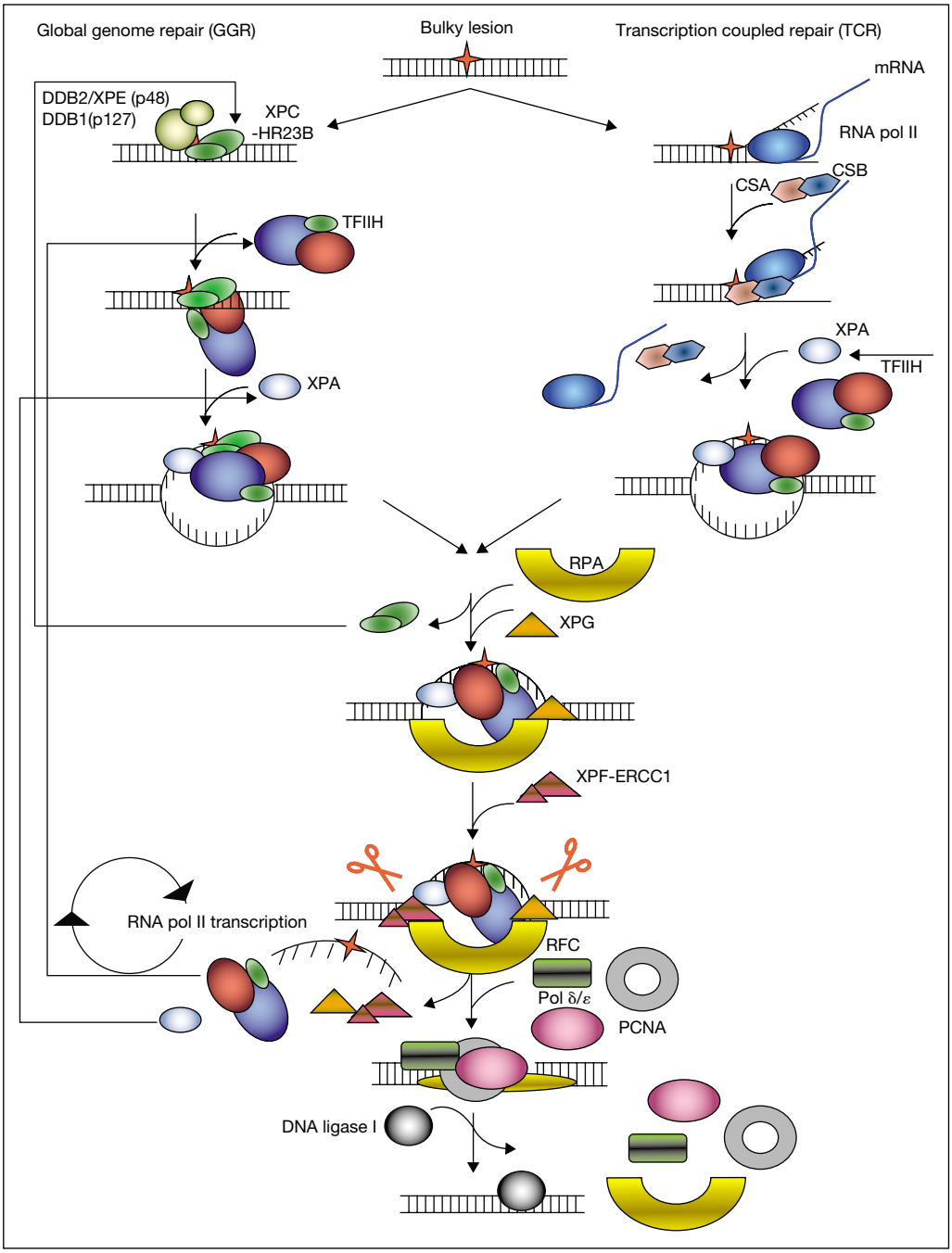
Recognition of DNA lesion upon global genome repair: Two protein complexes involved in the recognition step of NER have been identified. Each of them exhibits some specificity of recognition of either CPD or 6-4PP lesions. One of these complexes consists of the XPC protein complexed with HR23B (human homolog of the yeast RAD23 protein); the other complex called DDB factor (damage-DNA binding), is a heterodimer composed of the XPE (p48/DDB2) and of the p127/DDB1 proteins.

The XPC-HR23B complex exhibits strong and specific affinity for highly distorting DNA lesions, most notably 6-4PPs and cis-platin adducts (that are used for experimental purposes) [6, 7]. XPC-HR23B directly binds to the lesion [6, 8] and is essential for the formation of the incision complex. Conversely, affinity of XPC-HR23B is reduced in the case of lesions resulting in moderate DNA helix distortion, such as CPD [9]. In this case, recognition of the CPD lesion would rely on the DDB complex which is thought to enhance local DNA distortion, hence driving the recruitment of XPC-HR23B to the altered sequence [10, 11]. In rodent cell lines, efficiency of removal of CPD DNA lesions from the global genome is very poor. This is correlated with a barely detectable level of expression of the p48 subunit of the DDB complex [1, 12]. Very recently, however, Moser et al. have shown that the presence of DDB also enhanced repair of 6-4PP by facilitating recruitment of XPC-HR23B and subsequent factors [13]. Demarcation of the DNA distortion by XPC-HR23B results in local DNA unwinding and chromatin remodeling via activation of SWI/SNF factors [14], allowing subsequent loading of other repair factors to the damaged DNA sequence.

Recognition of DNA lesion upon transcription coupled repair: TCR is devoted to removal of DNA lesions lying in the transcribed strand of an active gene. In the case of TCR, the extent of DNA distortion does not seem to correlate with specific sets of recognition proteins [15]. Rather, stalling of RNA polymerase II in the course of transcription at the damaged sequence seems to initiate repair. Repair signaling depends on the Cockayne A and B (CS-A, CS-B) proteins the mutations of which result in TCR deficiency in Cockayne syndrome patients.

Subsequent Steps of DNA Repair

Once a DNA lesion has been detected specifically by either TCR or GGR complexes, subsequent steps of DNA repair obey a common repair pathway. Recently, more light was shed on the sequential steps of NER. J.M. Egly and co-workers set up an elegant system based on a mono-cisplatin-damaged DNA


Global genome repair (GGR)
Bulky lesion
Transcription coupled repair (TCR)
DDB2/XPE (p48)
DDB1(p127)
XPC
-HR23B
mRNA
RNA pol II
CSA
CSB
TFIIH
XPA
XPA
TFIIH
RPA
XPG
XPF-ERCC1
RNA pol II transcription
RFC
Pol δ/ε
PCNA
DNA ligase I

substrate (which mimics the DNA distortion induced by a 6-4PP) immobilized on magnetic beads. Sequential addition of purified NER factors allowed step by step reconstruction of NER and demonstration that most NER factors can be recycled [16] (fig. 1). Following entry of XPC/hHR23B, TFIIH was found to be the first protein recruited on the recognition complex. Then, in the presence of ATP, the XPB and XPD helicases that belong to the TFIIH complex unwind the damaged DNA around the lesion, yielding sequential association of XPA, RPA and XPG. The XPG protein is responsible for the 3′ incision at a distance of approximately 15–24 nucleotides from the lesion. Entry of XPG catalyzes release and recycling of the XPC-HR23B heteroduplex. Recruitment of XPF-ERCC1 triggers unilateral incision from the DNA lesion and release of XPA and TFIIH that are also recycled. In the absence of further DNA damage, the released form of TFIIH may then become available to join the RNA pol II transcription machinery. Entry of DNA resynthesis factors (DNA polymerases δ and ε, PCNA and RFC) promotes unloading of XPG and ERCC1-XPF from the DNA while RPA remains associated as part of DNA resynthesis apparatus. Finally, DNA ligase I carries out the ligation of the 5′-3′ ends of the synthesized DNA patch [17].

NER Deficiency Syndromes

Xeroderma Pigmentosum

Genetic heterogeneity: Xeroderma pigmentosum syndrome has been first described by Hebra and Kaposi in 1874. The clinical heterogeneity of XP patients has been suspected to result from genetic heterogeneity for a long time. Taking the absence of UDS (inherent to NER deficiency) and its recovery in case of functional complementation as a diagnostic criterion, investigators sought to determine genetic groups of XP complementation. Pioneer analyses of DNA repair by NER were carried out in heterocaryons following cell fusion. Seven XP groups of genetic complementation called XP-A to G could be

Fig. 1. Model for the NER mechanism: Upon global genome repair, bulky lesions are detected by XPC-HR23B assisted by DDB complex. Upon transcription-coupled NER, RNA pol II recognizes DNA damage when stalled at the site of a DNA lesion. CSA and CSB proteins make the lesion accessible to repair by displacing stalled RNA pol II. Then, the XPD and XPB ATP-dependent helicases that belong to TFIIH locally unwind the DNA around the lesion. This allows XPA and RPA to stabilize the open intermediate and to properly organize the dual incision apparatus around the lesion. XPG makes the 3′ DNA incision, whereas the XPF-ERCC1 complex is responsible for the 5′ cut in the damaged strand. After release of the lesion-containing oligonucleotide, the single strand gap is filled by DNA polymerase δ or ε in the presence of the replicating factors PCNA and RFC.

identified among cells isolated from patients with clinical XP [18]. These analyses also indicated the full NER capacity of cells from patients presenting the variant form of XP (XP-V). This is only recently that cells from XP-V patients (about 20% of all XPs) have been shown to suffer from unfaithful replicative translesion synthesis linked to mutation in the distributive POLη DNA polymerase [19, 20]. The full NER capacity of XP-V cells explains their nearly normal level of UV-survival and the delayed onset of photo-induced epithelioma in most XP-V patients compared to classical XP patients.

Frequency and geographic distribution of XP complementation groups is not uniform worldwide. In Europe and in North Africa the incidence of XP newborns is about 1:250,000 whereas in Japan incidence is 1:40 000 with a prevalence for XP-A complementation group (table 1).

Common hallmarks: Clinically, very high skin photosensitivity and a range of cutaneous afflictions of variable extent characterize all xeroderma pigmentosum patients. Onset of the disease occurs early in life with a mean age of 6–8 years. The first signs of the disease manifest in serious sunburn after minimal exposure to sunlight. Subsequently, erythema may persist for weeks following sunburn. Photosensitivity of XP patients is associated with skin abnormalities such as excessive freckling, hypo- and hyper-pigmentation and premature and accelerated skin aging. Skin surface is atrophic and dry, accounting for the name 'xeroderma'. Numerous precancerous lesions such as actinic keratoses (AK) and malignant skin tumors develop at early age with a preferential somatic distribution in photo-exposed skin. Skin tumors are mainly squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) that are both of keratinocyte origin. XP patients also develop malignant melanoma (MM), a melanocytic tumor. The first skin neoplasm in XP occurs at an average of 8 years, about 50 years earlier than that of the general population in the United States. In a historic study of a cohort of 830 XP patients, Ken Kraemer determined that the risk of developing skin tumors in young XP patients (under 20-year-olds) is 2000- to 4800-fold higher than in the general U.S. population [18, 21]. Molecular epidemiology studies have correlated this very high skin cancer proneness in XP patients with a high level of genomic mutations in tumor DNA. Sequences in key genes such as those encoding tumor suppressor proteins (p53, PATCHED) or oncogenes (RAS) have been found affected [22–26]. As a prototype tumor suppressor gene, *TP53* is mutated in 50 to 75% of XP skin cancers [27–29]. Mutations are preferentially located at pyrimidine-pyrimidine sequences (CC; CT; TC; TT) that are targets for UV-B induced 6-4PP and CPD DNA lesions. UV-induced mutations are essentially C to T transitions in non-XP tumors while CC to TT tandem transitions account for about 50% of mutations in XP tumors. These observations have suggested some differences in the mutagenic mechanism in non-XP versus in XP cells [30]. XP patients also develop non cutaneaous tumors such as astrocytomas,

Table 1. Principal characteristics of NER diseases

OMIM	Comple-mentation group[a]	Inheritance[b]	Percentage[c]	Tumor predis-position	Locus	Gene	Protein	Function	UV-sensitivity	GGR [%][d]	TCR [%][e]
278700	XP-A	AR	25*	++	9q22.3	*XPA*	XPA	Damaged DNA-binding interaction with TFIIH and XPF/XPG endonucleases	++++	<15	<15
133510	XP-B	AR	rare	+	2q21	*XPB/ ERCC3*	XPB	3′→5′ helicase in TFIIH	++	5–15	5–15
278720	XP-C	AR	25	+++	3p25.1	*XPC*	XPC	Damaged DNA-binding only involved in global genomic repair. Heterodimer with HHR23B	++	10	100
126340	XP-D	AR	15	++	19q13.2→ q13.3	*XPD/ ERCC2*	XPD	3′→5′ helicase in TFIIH	+++	30	30
600811	XP-E	AR	rare	+	11p12→p11	*DDB2*	XPE P48	Damaged DNA-binding only involved in global genomic repair. Heterodimer with DDB1	+	40–60	100
278760	XP-F	AR	6	++	16p13.3→ p13.13	*XPF/ ERCC4*	XPF	5′ structure-specific endonuclease heterodimer with ERCC1	++	10	10
133530	XP-G	AR	6	+++	13q32→q33	*XPG/ ERCC5*	XPG	3′ structure-specific endonuclease. Stabilization of the open complex	+++	5	5

Table 1. (continued)

OMIM	Complementation group[a]	Inheritance[b]	Percentage[c]	Tumor predisposition	Locus	Gene	Protein	Function	UV-sensitivity	GGR [%][d]	TCR [%][e]
603968	XP-V	AR	21	+	6p21.1	*POLH*	Pol h	Translesion DNA polymerase	+	100	100
216400	CS-A	AR	25	−	5q12.1	*CKN1/ ERCC8*	CSA	Transcription coupled repair	++	100	20–40
133540	CS-B	AR	75	−	10q11.23	*CKN2/ ERCC6*	CSB	Transcription coupled repair	++	100	20–40
608780	TTD-A	AR	rare	−	6p25.3	*GTF2H5*	TFB5/p8	Unknown	++	10–30	10–30
601675	TTD/XP-B	AR	rare	−	2q21	*XPB/ ERCC3*	XPB	3′→5′ helicase in TFIIH	+	40–60	40–60
278730	TTD/XP-D	AR	90	−	19q13.2?3	*XPD/ ERCC2*	XPD	3′→5′ helicase in TFIIH	++	20–60	20–60

[a]XP-A to XP-V: Xeroderma pigmentosum; CS-A and CS-B: Cockayne syndrome; TTD-A to TTD/XP-D: trichothiodystrophy.
[b]AR: autosomal recessive.
[c]From reference [75]; *: common in Japan, rare in the US and Europe.
[d]GGR: Global Genome Repair.
[e]TCR: Transcription Coupled Repair.

medulloblastomas, glioblastomas and malignant schwannomas, with a frequency about 10 times higher than in the general population [31]. The etiology of these internal tumors remains poorly understood.

Clinical heterogeneity: In addition to exacerbated signs of photosensitivity, XP-A, XP-D and XP-G patients may suffer with variable extent of developmental and neurological disorders [32, 33]. Neurological abnormalities occur progressively with age, resulting from the degeneration of neurons otherwise developed normally [32]. In some XP patients, expression of neurological problems may also be connected to abnormal development. These latter patients may present with microcephaly and mental retardation or dementia, which are associated with cortical atrophy and ventricular dilatation. Progressive deafness and loss of tendon stretch reflexes are considered as major criteria for the diagnosis of the neurologically affected XP patient [34]. The extreme form of XP with neurological problems has been termed 'DeSanctis-Cacchione syndrome' [32].

'Pure' XP Complementation Groups

XP-A: XP group A patients (XP-A) are afflicted by the most severe forms of the disease, including very high susceptibility toward photo-induced epithelioma, as well as severe mental retardation and progressive neurodegeneration. Most *XPA* mutations identified up to now lead to premature stop codon and protein truncation. Loss of XPA activity connected to protein truncation abolishes NER activity with respect to both GGR and TCR. Clinical impact of these truncating mutations is reflected by the expression of the most severe clinical features. Conversely, mutations that spare a minimal DNA binding region of the XPA protein result in milder clinical symptoms. XP-A cells are extremely sensitive to UV and exhibit very low residual levels of both GGR and TCR. As it has been shown in vitro that the XPA protein binds preferentially to UV damaged DNA compared to undamaged DNA, its biochemical function remains unclear. Physical interaction of XPA with the repair factors RPA, TFIIH and ERCC1 suggest its role in the correct positioning of the repair machinery around the lesion before dual DNA strand incisions [35–37]. More recently, XPA has been shown to interact with XAB1 (binding protein 1) and XAB2 (binding protein 2) whose function in NER remains unclear. XAB1 is a cytoplasmic GTPase that would participate in the nuclear localization of XPA [38]. XAB2 would play a role in TCR and in transcription [39].

XP-C: XP-C patients are highly photosensitive and cancer-prone but remain free of neurological problems. *XPC* mutations have been found spread out over the entire *XPC* gene. The majority of *XPC* mutations result in frameshift and protein truncation [40, 41]. In heterozygous carriers of a given *XPC* mutation (parents of XP-C patients), only the wild-type *XPC* mRNA was detected but not the mutated one [41]. In addition neither heterozygous

individuals ($XPC^{+/-}$) nor cultured $XPC^{+/-}$ cells isolated from these patients present phenotypic traits related to impaired NER capacity, suggesting that allele-specific expression of XPC might be finely controlled at both the transcriptional and post-transcriptional levels [41]. Interestingly, recent investigations have shown that XPC expression is upregulated at the transcriptional level in a p53-dependent manner upon UV-exposure [42]. Physiological impact of XPC truncations in skin has also been studied in our laboratory. We cultured primary keratinocytes and fibroblasts from XP-C patients, and for the first time, we were able to culture organotypic XP-C skin in vitro [43]. In the absence of UV exposure, organotypic XP-C skin disclosed yet undescribed alterations in the keratinocyte differentiation program, accompanied by increased numbers of Ki67 keratinocytes [43]. Importantly, most of these observations in organotypic cultures were confirmed in sections from freshly biopsied XP-C skin. Finally, organotypic combinations using XP-C fibroblasts within dermal equivalent provoked epidermal micro invasions of both normal and XP-C keratinocytes suggesting aberrant expression of matrix remodeling enzymes such as metalloproteinases in XP-C mesenchymal cells (Frechet et al., unpublished data).

The XPC protein is involved in numerous protein-protein interactions, which, in some instance, do not exhibit any obvious direct relationship with NER. XPC interacts with two proteins orthologous to the yeast Rad23 protein, HR23A and HR23B [44]. HR23A and HR23B have recently been shown to protect XPC against proteasomal degradation [45]. XPC has also been shown to functionally interact with the CENTRIN 2 (CEN2) protein, a centrosomal protein supporting HR23B-mediated XPC stabilization [46]. Other experiments showing interactions between XPC and TFIIH [47] have proposed it could act to recruit TFIIH to distorted DNA sequence [48].

XP-E: Compared to those from other groups of complementation, XP-E patients present features limited to cutaneous traits. Onset of skin tumors usually occurs later than 20 years of age. Recently, the gene responsible for the XP-E phenotype has been identified as DDB2, the smaller (48 kDa) subunit of the heterodimeric DDB (DNA damage binding) protein [49]. The biochemical function of DDB2/P48 has long been thought to be restricted to the role of an accessory factor in the recognition of CPD lesions during GGR. Mutation identified in P48 (DDB2) abolishes its physical interaction with P127 (DDB1) and hence complex stabilization and activity [49]. Mutations in DDB2 result in partial (about 50%) loss of NER capacity, explaining the relatively mild phenotype of XP-E patients. DDB2/P48 was recently found to be associated with the activation domain of the transcription factor E2F1. P48 also is part of a multi subunit complex including DDB1, the COP9 signalosome and the ubiquitin-ligase complex SCF-like [50]. Rapid degradation of DDB2 after UV has been correlated with its ubiquitination

by CULLIN4A, a subunit of the SCF-like complex [51]. Very recently, Sugasawa and colleagues demonstrated that DDB2 ubiquitination after UV exposure leads to XPC ubiquitination and potentiation of its binding to 6-4PP photoproducts [52]. Finally, transcription of the *DDB2* gene is regulated by P53, at both the basal level and following exposure to UV-radiations [11].

XP-F: Clinical traits of XP-F patients are also relatively mild, including photosensitivity, proneness to skin cancer and, in principle, no neurological problems. XPF is complexed with the ERCC1 protein, to form a stabilized heterodimeric complex exhibiting a structure-specific endonuclease activity [53, 54]. Mutations in *XPF* generally affect its C-terminal region and alter its interaction with ERCC1. Engineering of mouse with abolished XPF or ERCC1 expression has revealed its essential role since these mutations have far more important physiological impact compared to those with an impact limited to NER (for instance $XPA^{-/-}$ mutant mouse). In addition, XPF-ERCC1 has also been implicated in both removal of interstrand DNA cross-links, homology-mediated recombination and in immunoglobulin class switch recombination (CSR) as well. The role of XPF is not limited to NER but also extents to other DNA transactions.

Cockayne Syndrome

In its 'pure form' Cockayne syndrome (CS) can be caused by mutations, in either the *CKN1/CSA* (type I form) or *CKN2/ERCC6/CSB* (type II form) genes that correspond to the CS-A and CS-B complementation group representing 25% and 75% of CS patients respectively. Another form of CS called XP/CS cumulates features of both CS and XP and results from mutations in either one of the XP genes, *XPG*, *XPB* and *XPD*. About 180 cases of CS have been estimated worldwide with no preferential geographic distribution [55–57]. Clinical features of the CS may appear from birth to the 2nd to 3rd years of life. Type I (CS-A) form of CS is less severe than type II (CS-B). Life expectancy of CS-A patients can be up to young adult age (about 20 years) while vital prognostic is much more compromised in the case of CS-B patients. Interestingly, the type II/CS-B form of CS includes three clinical variants: UV-sensitive syndrome ($UV^{S}S$), the less severe form, cerebro-oculo-facio-skeletal syndrome (COFS), and the classic severe infantile variant [34]. Signs of poor growth, neurological abnormalities and premature aging generally characterize CS patients. Growth failure in these patients leads to 'cachectic dwarfism'. The loss of adipose tissue results in sunken eyes and beaked nose that confer characteristic bird-like face morphology. Neurological abnormalities are caused by progressive neurodegeneration and include delayed psychomotor development, mental retardation and microcephaly in most patients. Neuropathological examinations have shown multifocal patchy demyelinization in the cerebral and cerebellar cortex, dilated

ventricles and calcium deposits in basal ganglia and cerebral cortex. The pathology often reveals cataracts, dental caries and hearing loss. Patients with CS are sun sensitive and deficient in the removal of UV-induced DNA lesions by NER. However in contrast to XP patients, pure CS patients are not significantly prone to skin cancer compared to individuals from the general population.

CSA and CSB proteins: CSA and CSB proteins are required for the first steps of repair of transcribed genes. CSA is a 44-kDa protein containing five WD-40 repeats, involved in protein-protein interaction [58]. CSA was shown to translocate to the nuclear matrix and to co-localize in a CSB-dependent manner with the hyperphosphorylated form of RNA pol II during TCR [59]. Recently a protein complex exhibiting an ubiquitin ligase activity and composed of DDB1, cullin 4A, Roc1 and the COP9 signalosome has been shown to bind either CSA or DDB2 in the course of TCR and GGR, respectively, suggesting their qualitative and quantitative role in distinct DNA repair mechanisms [50].

CSB is a 168-kDa protein containing several ATPase motifs and a region homologous to the chromatin remodeling family of proteins, SWI/SNF2 [60]. The integrity of CSB is essential to TCR where it would help the transcriptional apparatus to pause, while repair is being processed and then, to restart. To fulfill this function, CSB probably facilitates physical chromatin opening, and through the recruitment of CSA-containing ubiquitin complex, clears the DNA from RNA polymerase II. It is not clear whether upon ubiquitination RNA polymerase returns to a back tracked position or is eliminated by degradation.

Trichothiodystrophy

About half of TTD patients are photosensitive. Mutations in either the *XPD*, *XPB* or *TTDA* gene are associated with photosensitive TTD and NER deficiency. Recently the locus *TTDN1* has been found associated with a group of non-photosensitive TTD patients whose cells exhibit normal level of NER [61].

Clinical features of patients with TTD are highly variable in expression and severity. Brittle hair with low sulfure content is a common characteristic of TTD patients but this trait may be shared with non-TTD hypotrichoses. Heterogeneity of symptoms and rarity of TTD patients has led to multiple names or acronyms of the disease. For example, Baden et al. used the acronym BIDS for: Brittle-hair, Intellectual impairment, Decreased fertility, and Short stature in association with low-sulfure content in hair [62]. Jorizzo et al. suggested the term IBIDS for Ichthyosis-associated cases in conjunction with low-sulfure content in hair of BIDS [63] and Crovato et al. proposed PIBIDS for photosensitivity associated with IBIDS [64].

TTDA: TFB5 was recently identified as the deficient protein in patients falling in the TTD-A complementation group. TFB5 was first identified in yeast as a core component of general transcription and DNA repair factor IIH

[65]. Giglia-Mari et al. cloned the human cDNA corresponding to the *GTF2H5* gene from primary fibroblasts. Transfection of the corresponding cDNA restored normal level of repair in TTD-A cells suggesting that the *GTF2H5* gene is mutated in TTD-A [66]. TFB5 transiently regulates steady-state levels of TFIIH, including in WT cells. TFB5 is thought to act at the posttranscriptional level, perhaps in a chaperone-like manner contributing to complex assembly and/or maintenance [66]. Conversely, absence of TFB5 in TTD-A cells would alter stability of TFIIH and its repair and transcriptional activities.

Combined Complementation Groups

XP-G: Determination of mutations in the *XPG* gene has revealed some genotype-phenotype correlations. Miscoding *XPG* mutations compatible with the production of a full-length protein result in the 'pure' XP-G syndrome. Conversely, truncated and/or unstable XPG is associated with the composite XP-G/CS syndrome. About 50% of the 24 identified XP-G patients also present some characteristic features of CS [67]. *XPG* mRNA encodes a 133-kDa protein with a single-stranded 3′-DNA endonuclease activity. Correct positioning of the XPG protein conditions efficiency of the 5′ incision by ERCC1-XPF.

XP-B and XP-D: XPB and XPD proteins exert ATP-dependent 3′-5′ and 5′-3′ DNA helicase activity, respectively. Both of them are components of the TFIIH transcription factor and contribute to both transcription and NER. Patients with *XPB* mutations are extremely rare. There is only one family with *XPB* mutations leading to the TTD/XP-B syndrome, and two other presenting XP-B/CS features. In contrast, no 'pure XP-B' patients have been identified to date. Rarity of the *XPB* mutations suggests pivotal roles of the WT protein in development and viability, presumably through the transcription of essential genes. In contrast, mutations in the *XPD* gene are much more numerous than in the *XPB* gene, perhaps because its role in transcription is less crucial than that of *XPB*. *XPD* mutations are spread out all over the *XPD* gene and most of them give rise to miscoding mRNA. Some mutations in the *XPD* gene constitute hotspots that can be specifically responsible for the TTD, Cockayne or XP syndrome. Distinct mutational hotspots located in the amino- versus carboxy-terminal ends of XPD, respectively, may affect different functional protein domains but result in a similar phenotype. For instance, the R112H, the R658C, or the R722W mutations constitute frequent and characteristic hotspots of the TTD/XP-D syndrome. In turn, the R683W XPD mutation, which is also located in the carboxy-terminal helicase domain of the protein, is found in about 75% of XP-D patients [68, 69]. Functional analyses in vitro have shown that helicase activity of XPD is essential for NER but not for transcription [70]. Mutations in the N-terminal region of XPD affect the intrinsic helicase activity [71]. Mutations in the C-terminal region of XPD impair its interaction with the p44 subunit of TFIIH, resulting in

decreased helicase activity [70]. It has been proposed that *XPD* mutations affecting NER activity would result in the photosensitive/cancer-prone XP-D syndrome while *XPD* mutations altering basal transcription would rather lead to phenotypes associated with the TTD/XP-D or the CS/XP-D syndrome [69].

Therapy

Very strict photoprotection remains the only efficient treatment proposed to photosensitive NER-deficient patients. K. Kraemer conducted a 3-year prospective study using oral isotretinoin (a synthetic derivative of vitamin A) in five XP patients. A significant decrease (63%) in skin cancer incidence was observed upon treatment. However, tumoral burst was observed by 6 months following treatment discontinuation [72]. Another clinical trial directed by Dan Yarosh relied on the use of the bacterial enzyme T4 endonuclease V which removes CPD DNA lesions. Topical application of a liposome formulation of this enzyme over a period of one year resulted in significant decrease in the number of BCC and AK in treated patients of age <18 [73]. Finally, engraftment of epithelial sheets obtained from genetically corrected keratinocytes ex vivo would provide another means of improving life conditions of candidate XP patients. To this end, we could recently correct the phenotype of several primary strains of XP-C keratinocytes ex vivo using retroviral vectors expressing the *XPC* gene [74]. Our effort is now devoted to corrective gene transfer in keratinocytes of patients using procedures compatible with skin graft perspectives.

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References

1 Bohr VA, Smith CA, Okumoto DS, Hanawalt PC: DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell 1985;40:359–369.

2 Svejstrup JQ, Wang Z, Feaver WJ, Wu X, Bushnell DA, Donahue TF, Friedberg EC, Kornberg RD: Different forms of TFIIH for transcription and DNA repair: holo-TFIIH and a nucleotide excision repairosome. Cell 1995;80:21–28.

3 Maldonado E, Shiekhattar R, Sheldon M, Cho H, Drapkin R, Rickert P, Lees E, Anderson CW, Linn S, Reinberg D: A human RNA polymerase II complex associated with SRB and DNA-repair proteins. Nature 1996;381:86–89.
4 Guzder SN, Sung P, Prakash L, Prakash S: Nucleotide excision repair in yeast is mediated by sequential assembly of repair factors and not by a pre-assembled repairosome. J Biol Chem 1996;271:8903–8910.
5 Seeberg E, Fuchs RP: Acetylaminofluorene bound to different guanines of the sequence -GGCGCC- is excised with different efficiencies by the UvrABC excision nuclease in a pattern not correlated to the potency of mutation induction. Proc Natl Acad Sci USA 1990;87:191–194.
6 Sugasawa K, Ng JM, Masutani C, Iwai S, van der Spek PJ, Eker AP, Hanaoka F, Bootsma D, Hoeijmakers JH: Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. Mol Cell 1998;2:223–232.
7 Hey T, Lipps G, Sugasawa K, Iwai S, Hanaoka F, Krauss G: The XPC-HR23B complex displays high affinity and specificity for damaged DNA in a true-equilibrium fluorescence assay. Biochemistry 2002;41:6583–6587.
8 Volker M, Mone MJ, Karmakar P, van Hoffen A, Schul W, Vermeulen W, Hoeijmakers JH, van Driel R, van Zeeland AA, Mullenders LH: Sequential assembly of the nucleotide excision repair factors in vivo. Mol Cell 2001;8:213–224.
9 Sugasawa K, Ng JM, Masutani C, Maekawa T, Uchida A, van der Spek PJ, Eker AP, Rademakers S, Visser C, Aboussekhra A, Wood RD, Hanaoka F, Bootsma D, Hoeijmakers JH: Two human homologs of Rad23 are functionally interchangeable in complex formation and stimulation of XPC repair activity. Mol Cell Biol 1997;17:6924–6931.
10 Wakasugi M, Kawashima A, Morioka H, Linn S, Sancar A, Mori T, Nikaido O, Matsunaga T: DDB accumulates at DNA damage sites immediately after UV irradiation and directly stimulates nucleotide excision repair. J Biol Chem 2002;277:1637–1640.
11 Fitch ME, Nakajima S, Yasui A, Ford JM: In vivo recruitment of XPC to UV-induced cyclobutane pyrimidine dimers by the *DDB2* gene product. J Biol Chem 2003;278:46906–46910.
12 Vreeswijk MP, van Hoffen A, Westland BE, Vrieling H, van Zeeland AA, Mullenders LH: Analysis of repair of cyclobutane pyrimidine dimers and pyrimidine 6-4 pyrimidone photoproducts in transcriptionally active and inactive genes in Chinese hamster cells. J Biol Chem 1994;269:31858–31863.
13 Moser J, Volker M, Kool H, Alekseev S, Vrieling H, Yasui A, van Zeeland AA, Mullenders LH: The UV-damaged DNA binding protein mediates efficient targeting of the nucleotide excision repair complex to UV-induced photo lesions. DNA Repair (Amst) 2005;4:571–582.
14 Hara R, Sancar A: The SWI/SNF chromatin-remodeling factor stimulates repair by human excision nuclease in the mononucleosome core particle. Mol Cell Biol 2002;22:6779–6787.
15 van Hoffen A, Venema J, Meschini R, van Zeeland AA, Mullenders LH: Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts. EMBO J 1995;14:360–367.
16 Riedl T, Hanaoka F, Egly JM: The comings and goings of nucleotide excision repair factors on damaged DNA. EMBO J 2003;22:5293–5303.
17 Tomkinson AE, Levin DS: Mammalian DNA ligases. Bioessays 1997;19:893–901.
18 Cleaver JE, Thompson LH, Richardson AS, States JC: A summary of mutations in the UV-sensitive disorders: xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy. Hum Mutat 1999;14:9–22.
19 Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M, Araki M, Iwai S, Takio K, Hanaoka F: The *XPV* (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. Nature 1999;399:700–704.
20 Johnson RE, Kondratick CM, Prakash S, Prakash L: hRAD30 mutations in the variant form of xeroderma pigmentosum. Science 1999;285:263–265.
21 Kraemer KH: Sunlight and skin cancer: another link revealed. Proc Natl Acad Sci USA 1997;94: 11–14.
22 Steingrimsdottir H, Beare D, Carr AM, Cole J, Lehmann AR: U.v.-hypermutability of xeroderma pigmentosum cells demonstrated with a DNA-based mutation system. Oncogene 1995;10:2057–2066.

23 Sato M, Nishigori C, Zghal M, Yagi T, Takebe H: Ultraviolet-specific mutations in p53 gene in skin tumors in xeroderma pigmentosum patients. Cancer Res 1993;53:2944–2946.
24 D'Errico M, Calcagnile A, Canzona F, Didona B, Posteraro P, Cavalieri R, Corona R, Vorechovsky I, Nardo T, Stefanini M, Dogliotti E: UV mutation signature in tumor suppressor genes involved in skin carcinogenesis in xeroderma pigmentosum patients. Oncogene 2000;19:463–467.
25 Daya-Grosjean L, Robert C, Drougard C, Suarez H, Sarasin A: High mutation frequency in ras genes of skin tumors isolated from DNA repair deficient xeroderma pigmentosum patients. Cancer Res 1993;53:1625–1629.
26 Bodak N, Queille S, Avril MF, Bouadjar B, Drougard C, Sarasin A, Daya-Grosjean L: High levels of patched gene mutations in basal-cell carcinomas from patients with xeroderma pigmentosum. Proc Natl Acad Sci USA 1999;96:5117–5122.
27 Spatz A, Giglia-Mari G, Benhamou S, Sarasin A: Association between DNA repair-deficiency and high level of p53 mutations in melanoma of Xeroderma pigmentosum. Cancer Res 2001;61: 2480–2486.
28 Giglia G, Dumaz N, Drougard C, Avril MF, Daya-Grosjean L, Sarasin A: p53 mutations in skin and internal tumors of xeroderma pigmentosum patients belonging to the complementation group C. Cancer Res 1998;58:4402–4409.
29 Dumaz N, Drougard C, Sarasin A, Daya-Grosjean L: Specific UV-induced mutation spectrum in the p53 gene of skin tumors from DNA-repair-deficient xeroderma pigmentosum patients. Proc Natl Acad Sci USA 1993;90:10529–10533.
30 Sarasin A: The molecular pathways of ultraviolet-induced carcinogenesis. Mutat Res 1999;428: 5–10.
31 Nakamura T, Ono T, Yoshimura K, Arao T, Kondo S, Ichihashi M, Matsumoto A, Fujiwara Y: Malignant schwannoma associated with xeroderma pigmentosum in a patient belonging to complementation group D. J Am Acad Dermatol 1991;25:349–353.
32 Robbins JH, Brumback RA, Mendiones M, Barrett SF, Carl JR, Cho S, Denckla MB, Ganges MB, Gerber LH, Guthrie RA, et al: Neurological disease in xeroderma pigmentosum. Documentation of a late onset type of the juvenile onset form. Brain 1991;114 (Pt 3):1335–1361.
33 Robbins JH: Xeroderma pigmentosum. Defective DNA repair causes skin cancer and neurodegeneration. JAMA 1988;260:384–388.
34 Rapin I, Lindenbaum Y, Dickson DW, Kraemer KH, Robbins JH: Cockayne syndrome and xeroderma pigmentosum. Neurology 2000;55:1442–1449.
35 Wakasugi M, Sancar A: Order of assembly of human DNA repair excision nuclease. J Biol Chem 1999;274:18759–18768.
36 Li L, Lu X, Peterson CA, Legerski RJ: An interaction between the DNA repair factor XPA and replication protein A appears essential for nucleotide excision repair. Mol Cell Biol 1995;15: 5396–5402.
37 de Laat WL, Jaspers NG, Hoeijmakers JH: Molecular mechanism of nucleotide excision repair. Genes Dev 1999;13:768–785.
38 Nitta M, Saijo M, Kodo N, Matsuda T, Nakatsu Y, Tamai H, Tanaka K: A novel cytoplasmic GTPase XAB1 interacts with DNA repair protein XPA. Nucleic Acids Res 2000;28:4212–4218.
39 Nakatsu Y, Asahina H, Citterio E, Rademakers S, Vermeulen W, Kamiuchi S, Yeo JP, Khaw MC, Saijo M, Kodo N, Matsuda T, Hoeijmakers JH, Tanaka K: XAB2, a novel tetratricopeptide repeat protein involved in transcription-coupled DNA repair and transcription. J Biol Chem 2000;275: 34931–34937.
40 Li L, Bales ES, Peterson CA, Legerski RJ: Characterization of molecular defects in xeroderma pigmentosum group C. Nat Genet 1993;5:413–417.
41 Chavanne F, Broughton BC, Pietra D, Nardo T, Browitt A, Lehmann AR, Stefanini M: Mutations in the *XPC* gene in families with xeroderma pigmentosum and consequences at the cell, protein, and transcript levels. Cancer Res 2000;60:1974–1982.
42 Adimoolam S, Ford JM: p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene. Proc Natl Acad Sci USA 2002;99:12985–12990.
43 Bernerd F, Asselineau D, Vioux C, Chevallier-Lagente O, Bouadjar B, Sarasin A, Magnaldo T: Clues to epidermal cancer proneness revealed by reconstruction of DNA repair-deficient xeroderma pigmentosum skin in vitro. Proc Natl Acad Sci USA 2001;98:7817–7822.

44 van der Spek PJ, Eker A, Rademakers S, Visser C, Sugasawa K, Masutani C, Hanaoka F, Bootsma D, Hoeijmakers JH: XPC and human homologs of RAD23: intracellular localization and relationship to other nucleotide excision repair complexes. Nucleic Acids Res 1996;24:2551–2559.
45 Ng JM, Vermeulen W, van der Horst GT, Bergink S, Sugasawa K, Vrieling H, Hoeijmakers JH: A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. Genes Dev 2003;17:1630–1645.
46 Araki M, Masutani C, Takemura M, Uchida A, Sugasawa K, Kondoh J, Ohkuma Y, Hanaoka F: Centrosome protein centrin 2/caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. J Biol Chem 2001;276:18665–18672.
47 Araujo SJ, Nigg EA, Wood RD: Strong functional interactions of TFIIH with XPC and XPG in human DNA nucleotide excision repair, without a preassembled repairosome. Mol Cell Biol 2001;21:2281–2291.
48 Evans E, Moggs JG, Hwang JR, Egly JM, Wood RD: Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. EMBO J 1997;16:6559–6573.
49 Rapic-Otrin V, Navazza V, Nardo T, Botta E, McLenigan M, Bisi DC, Levine AS, Stefanini M: True XP group E patients have a defective UV-damaged DNA binding protein complex and mutations in DDB2 which reveal the functional domains of its p48 product. Hum Mol Genet 2003;12:1507–1522.
50 Groisman R, Polanowska J, Kuraoka I, Sawada J, Saijo M, Drapkin R, Kisselev AF, Tanaka K, Nakatani Y: The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell 2003;113:357–367.
51 Matsuda N, Azuma K, Saijo M, Iemura S, Hioki Y, Natsume T, Chiba T, Tanaka K, Tanaka K: DDB2, the xeroderma pigmentosum group E gene product, is directly ubiquitylated by Cullin 4A-based ubiquitin ligase complex. DNA Repair (Amst) 2005;4:537–545.
52 Sugasawa K, Okuda Y, Saijo M, Nishi R, Matsuda N, Chu G, Mori T, Iwai S, Tanaka K, Tanaka K, Hanaoka F: UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. Cell 2005;121:387–400.
53 Sijbers AM, de Laat WL, Ariza RR, Biggerstaff M, Wei YF, Moggs JG, Carter KC, Shell BK, Evans E, de Jong MC, Rademakers S, de Rooij J, Jaspers NG, Hoeijmakers JH, Wood RD: Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell 1996;86:811–822.
54 Park CH, Mu D, Reardon JT, Sancar A: The general transcription-repair factor TFIIH is recruited to the excision repair complex by the XPA protein independent of the TFIIE transcription factor. J Biol Chem 1995;270:4896–4902.
55 Nance MA, Berry SA: Cockayne syndrome: review of 140 cases. Am J Med Genet 1992;42: 68–84.
56 Mahmoud AA, Yousef GM, Al-Hifzi I, Diamandis EP: Cockayne syndrome in three sisters with varying clinical presentation. Am J Med Genet 2002;111:81–85.
57 Colella S, Nardo T, Mallery D, Borrone C, Ricci R, Ruffa G, Lehmann AR, Stefanini M: Alterations in the *CSB* gene in three Italian patients with the severe form of Cockayne syndrome (CS) but without clinical photosensitivity. Hum Mol Genet 1999;8:935–941.
58 Henning KA, Li L, Iyer N, McDaniel LD, Reagan MS, Legerski R, Schultz RA, Stefanini M, Lehmann AR, Mayne LV, Friedberg EC: The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. Cell 1995;82:555–564.
59 Kamiuchi S, Saijo M, Citterio E, de Jager M, Hoeijmakers JH, Tanaka K: Translocation of Cockayne syndrome group A protein to the nuclear matrix: possible relevance to transcription-coupled DNA repair. Proc Natl Acad Sci USA 2002;99:201–206.
60 Troelstra C, Odijk H, de Wit J, Westerveld A, Thompson LH, Bootsma D, Hoeijmakers JH: Molecular cloning of the human DNA excision repair gene ERCC-6. Mol Cell Biol 1990;10:5806–5813.
61 Nakabayashi K, Amann D, Ren Y, Saarialho-Kere U, Avidan N, Gentles S, MacDonald JR, Puffenberger EG, Christiano AM, Martinez-Mir A, Salas-Alanis JC, Rizzo R, Vamos E, Raams A, Les C, Seboun E, Jaspers NG, Beckmann JS, Jackson CE, Scherer SW: Identification of *C7orf11* (*TTDN1*) gene mutations and genetic heterogeneity in nonphotosensitive trichothiodystrophy. Am J Hum Genet 2005;76:510–516.

62 Baden HP, Jackson CE, Weiss L, Jimbow K, Lee L, Kubilus J, Gold RJ: The physicochemical properties of hair in the BIDS syndrome. Am J Hum Genet 1976;28:514–521.
63 Jorizzo JL, Atherton DJ, Crounse RG, Wells RS: Ichthyosis, brittle hair, impaired intelligence, decreased fertility and short stature (IBIDS syndrome). Br J Dermatol 1982;106:705–710.
64 Crovato F, Borrone C, Rebora A: Trichothiodystrophy – BIDS, IBIDS and PIBIDS? Br J Dermatol 1983;108:247.
65 Ranish JA, Hahn S, Lu Y, Yi EC, Li XJ, Eng J, Aebersold R: Identification of TFB5, a new component of general transcription and DNA repair factor IIH. Nat Genet 2004;36:707–713.
66 Giglia-Mari G, Coin F, Ranish JA, Hoogstraten D, Theil A, Wijgers N, Jaspers NG, Raams A, Argentini M, van der Spek PJ, Botta E, Stefanini M, Egly JM, Aebersold R, Hoeijmakers JH, Vermeulen W: A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat Genet 2004;36:714–719.
67 Clarkson SG: The XPG story. Biochimie 2003;85:1113–1121.
68 Taylor EM, Broughton BC, Botta E, Stefanini M, Sarasin A, Jaspers NG, Fawcett H, Harcourt SA, Arlett CF, Lehmann AR: Xeroderma pigmentosum and trichothiodystrophy are associated with different mutations in the *XPD* (*ERCC2*) repair/transcription gene. Proc Natl Acad Sci USA 1997;94:8658–8663.
69 Lehmann AR: The xeroderma pigmentosum group D (*XPD*) gene: one gene, two functions, three diseases. Genes Dev 2001;15:15–23.
70 Coin F, Marinoni JC, Rodolfo C, Fribourg S, Pedrini AM, Egly JM: Mutations in the XPD helicase gene result in XP and TTD phenotypes, preventing interaction between XPD and the p44 subunit of TFIIH. Nat Genet 1998;20:184–188.
71 Dubaele S, Proietti De Santis L, Bienstock RJ, Keriel A, Stefanini M, Van Houten B, Egly JM: Basal transcription defect discriminates between xeroderma pigmentosum and trichothiodystrophy in XPD patients. Mol Cell 2003;11:1635–1646.
72 Kraemer KH, DiGiovanna JJ, Moshell AN, Tarone RE, Peck GL: Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin. N Engl J Med 1988;318:1633–1637.
73 Yarosh D, Klein J, O'Connor A, Hawk J, Rafal E, Wolf P: Effect of topically applied T4 endonuclease V in liposomes on skin cancer in xeroderma pigmentosum: a randomised study. Xeroderma Pigmentosum Study Group. Lancet 2001;357:926–929.
74 Arnaudeau-Bégard C, Brellier F, Chevallier-Lagente O, Hoeijmakers JH, Bernerd F, Sarasin A, Magnaldo T: Genetic correction of DNA repair deficient/cancer prone xeroderma pigmentosum group C keratinocytes. Hum Gene Ther 2003;14:983–996.
75 Kraemer KH: Heritable diseases with increased sensitivity to cellular injury, in Fredberg IM, Eisen AZ, et al (eds): Fitzpatrick's Dermatology in General Medecine, 6th ed. McGraw-Hill, New York, 2003.

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Oxidative Damage to DNA in Non-Malignant Disease: Biomarker or Biohazard?

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Abstract

Oxidative damage to DNA has been examined in many non-malignant conditions, in most cases for its utility as a marker of oxidative stress. Whilst this may prove useful, attempts to answer the question – why might oxidative damage be important in this disease? – would provide added value to the biomarker data, as well as give clues to pathogenesis and perhaps therapy. In this chapter, data from the scientific literature are considered broadly, where oxidative damage to DNA has been analysed either in tissues or in extracellular matrices, such as urine, in various groups of non-malignant disease. The lesion of primary focus is 8-hydroxy-7,8-dihydro-2′-deoxyguanosine, only because this is the most widely measured lesion. By coupling biomarker information with the characteristics of the disease and a set of general mechanisms whereby DNA oxidation may be pathogenic (retrospectively derived from the literature examined), we can ascribe pathogenic roles for DNA oxidation in various diseases. Based on available experimental evidence, for a wide range of conditions, such mechanisms would include prominent roles for the induction of mitochondrial dysfunction, promotion of cytotoxicity and modulation of inflammatory responses. Our general conclusion is that, dependent on the disease, oxidative DNA damage may be a biomarker, biohazard or both of these.

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Reactive oxygen species (ROS) are ubiquitous by-products of aerobic metabolism and comprise a group of free radical and non-radical species, e.g. superoxide, hydrogen peroxide, hydroxyl radical and hypohalous acids, of varying reactivity. Although ROS have a number of defined physiological functions, it is their potentially detrimental effects, specifically on DNA, that are the focus of this chapter. There are numerous instances throughout the scientific literature

where the origins of ROS, both endogenous and exogenous, have been described [1]. Similarly, the identities, mechanisms of formation and repair of products of DNA oxidation have recently been comprehensively reviewed [2].

Conceptually, it is straightforward to appreciate a role for DNA oxidation in the pathogenesis of cancer, for example via the induction of mutations through the mis-coding properties of DNA lesions. However, there still seems to be a lack of experimental evidence directly linking DNA oxidation and carcinogenesis. The links between chronic exposure to oxidants and carcinogenesis, of which DNA oxidation is a component, are a little firmer. Certain, initially non-malignant, chronic inflammatory conditions are associated with significant potential to progress to malignancy, and aside from the modulation of gene expression, chronic inflammation is likely to involve elevated production of ROS and increased oxidative damage to DNA. Examples of this scenario include associations between inflammatory bowel diseases, such as ulcerative colitis, Crohn's disease and colorectal cancer; *H. pylori* infection/gastritis and gastric cancer; chronic gastric reflux and oesophageal cancer; cirrhosis, hepatitis B/C infection and hepatocellular carcinoma. In fact, it would seem that chronic inflammation can drive malignancy in several tissues and elevated levels of DNA oxidation products have been detected in both inflamed and malignant tissue.

It seems that, initially at least, there is a more tenuous link between DNA oxidation and the pathogenesis of non-malignant conditions, despite the fact that markers of oxidative damage to DNA have been measured in many examples of this type of disease. Is it simply that some of these markers serve as convenient, established, markers of oxidative stress (an imbalance between oxidants and antioxidants in favour of the former)? Or does their occurrence imply something about a pathogenic role for oxidative DNA damage in these conditions? If we consider that oxidative DNA damage does have a pathogenic role in non-malignant disease, what general mechanisms may operate? (i), Damage to the genome may be so great that cells are unable to function, resulting in cytotoxicity, which could lead to the selective deletion of irreplaceable cell populations, e.g. in the CNS and/or pancreatic islet cells. Associated with this is more specific damage to mitochondria and the mitochondrial genome, leading to deficits in ATP production with consequences for mitochondrial/nuclear DNA repair and increased production of ROS, ultimately leading to compromised cell function and cell death. An associated factor, related to cell death is the induction of premature senescence in selected cell populations, as there is some evidence that oxidative damage can accelerate telomere shortening [3]. (ii), The cytotoxic impact of DNA damage may lead to release of cell components into the extracellular medium, this may become important in conditions where damaged material cannot be scavenged effectively, e.g. in autoimmune diseases like systemic lupus erythematosus (SLE). (iii), DNA damage

may be sufficiently selective that coding regions of expressed genes are specifically damaged, or promoter regions, or epigenetic processes such as DNA methylation are affected leading to alterations in gene expression. The impact of oxidative DNA damage on promoter function, for example, is relatively under-studied yet some more recent studies would suggest that this represents a new dimension to the pathogenic importance of oxidative DNA damage extending beyond mutagenicity [3, 4].

Measurement and Importance of Oxidative Damage to DNA in Non-Malignant Disease

A plethora of DNA lesions arising from oxidative processes have been reported in the literature, encompassing various base and sugar modifications, strand breaks and more complex lesions, such as the bulkier adducts formed from the interaction of reactive aldehydes, derived from lipid peroxidation, with DNA [2]. Of this array of lesions, relatively few have received intense scrutiny, even in malignant disease. Of the simpler adducts, 8-hydroxy-7,8-dihydro-2′-deoxyguanosine (8-OH-dG) has received by far the most attention, despite elements of the analysis being fraught with problems, largely confined to its examination in extracted cellular DNA [5]. Deficits in the analysis of other lesions may have arisen because of the well-established literature for the analysis of 8-OH-dG, prompting investigators to focus upon this lesion at the expense of others, coupled with an apparent ease of 8-OH-dG formation/abundance. Additionally, despite attempts to search for and analyse multiple lesions, 8-OH-dG became the lesion of choice as few methodologies can detect multiple lesions. Levels of 8-OH-dG analysed in extracellular fluids, by chromatographic methods or immunoassay, have also received much attention, although the interpretation of changes or differences in these levels is not entirely straightforward. This is partly due to the fact that elevated levels in urine or other extracellular matrices, could indicate enhanced oxidative damage to the genome, increased repair of lesions or even contributions from the diet and cell turnover. Notable progress is being made regarding the interpretation of extracellular levels of 8-OH-dG, with an emerging view that diet and cell turnover may have little role in contributing to urinary 8-OH-dG levels [6]. With this in mind, there are still several potential intracellular sources (repair activities) for this lesion, but if diet and cell turnover can be excluded, this presents an opportunity to re-interpret existing data in light of this new information. Whether these emerging findings are applicable to other urinary lesions derived from oxidative DNA damage (e.g. thymine glycol, 5-hydroxymethyluracil) remains to be fully established [7, 8].

There have been notably fewer studies measuring lesions derived from DNA oxidation other than 8-OH-dG in disease, thus their biological role in this context is less well understood. Depending upon the particular conditions of DNA oxidation, the pattern of lesions produced varies, therefore, whilst 8-OH-dG may be the most frequently studied, it is not necessarily the most abundant lesion under all circumstances. Furthermore, other lesions have potentially important biological consequences, and at a more basic level the potential effects of some of these lesions on mutational events, genome integrity, DNA replication and control of gene expression have received some study. Many of these lesions, which, for the purposes of this discussion include the formamidopyrimidines, thymine glycol, 5-hydroxymethyluracil (5-OHMeUra), 5-formyluracil and 8,5′-cyclo-2′-deoxyadenosine (cyclo-dA), have been shown to possess mutagenic properties, but their effects can extend beyond this. Thymine glycol, while possessing some, albeit weak, mutagenic potential has the ability to block DNA replication, and could therefore contribute to cytostasis and cell death. Weak replication blocking ability is also shown by 5-formyluracil, formed from oxidative modification of the methyl group in thymine. There are two routes to the formation of 5-OHMeUra, and its potential biological effect is dependent upon the identity of the base from which it is formed (thymine or 5-methylcytosine). Firstly, oxidation of the thymine methyl group to yield 5-OHMeUra could have mutagenic consequences; additionally 5-OHMeUra can induce large or intermediate deletions in the genome. The formation of 5-OHMeUra via oxidation/deamination of 5-methylcytosine in CpG islands could modify gene expression, by interfering with the activities of sequence-specific DNA binding proteins involved in controlling gene expression via DNA methylation. It would seem that several different DNA oxidation products have the potential to modulate gene expression by affecting the ability of transcription factors to bind to promoter regions. 5-Formyluracil, 5-OHMeUra, cyclo-dA and 8-OH-dG have variously been shown to affect the DNA-binding properties of NFκB, Sp1 and AP-1, extending the potential pathogenic importance of these lesions beyond mutation [3, 9].

In terms of the sub-cellular sources of lesions, the nucleus, deoxynucleotide pools and mitochondria are logical locations. Mitochondrial DNA would be a particular target of interest largely because, although mitochondria are endowed with their own set of repair enzymes, mitochondrial DNA is more exposed than nuclear DNA and lies close to an important site for potential ROS production. The detrimental impact of damage to mitochondrial DNA is particularly acute in those cells dependent on mitochondrial function, such as neuronal cells or cardiomyocytes, and indeed studies would indicate a role for such damage in the pathogenesis of Alzheimer's disease or heart failure, for example [10]. Figure 1 considers the potential sources of DNA oxidation products.

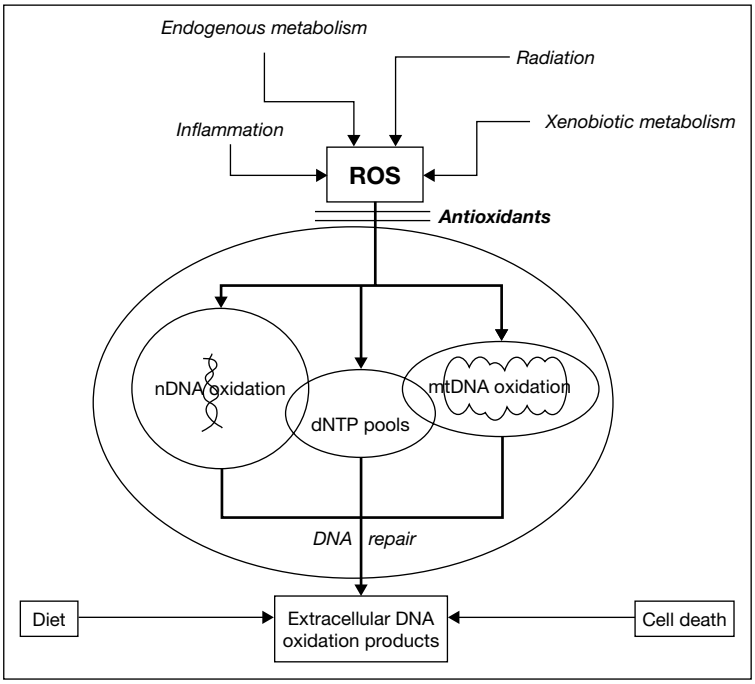

Fig. 1. Potential sources and targets of intra- and extracellular DNA oxidation products. Antioxidants can counteract DNA oxidation and include low molecular weight species, e.g. vitamin C, vitamin E and glutathione, as well as high molecular weight antioxidants, e.g. catalase, glutathione peroxidase and superoxide dismutase. 2′-deoxynucleotide triphosphates oxidised in the nucleotide (dNTP) pools act as a potential source for mis-incorporation of oxidation products into DNA, 8-hydroxy-2′-deoxyguanosine triphosphate, for example, can be removed from the cell via the action of human mutT homologue (hMTH1). nDNA: nuclear DNA; mtDNA: mitochondrial DNA.

As an analytical target, the popularity of 8-OH-dG relies on an abundant literature concerning analysis and biological properties, as well as the importance ascribed by the presence of multiple DNA repair pathways for this lesion. Whether the repair pathways are extensive simply because they have been studied more closely or because this lesion is peculiarly genotoxic is not entirely clear. Certainly, 8-OH-dG has been widely analysed in tissues and extracellular matrices in a number of non-malignant conditions, although a limited number of studies have examined more than one lesion. Thus, a major focus of this chapter is on 8-OH-dG, simply because of the relatively abundant literature devoted to it, a situation not shared by most of the other lesions resulting from DNA oxidation. In the following sections, we discuss a range of non-malignant

conditions and also refer the reader to a recent review article which considers many of the conditions discussed herein and contains numerous literature citations relating to particular diseases [2].

Autoimmune Disease

Inflammation is an important component of the pathology of systemic and tissue-specific autoimmune diseases, e.g. rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis, Sjogren's syndrome, Behcet's disease. The majority of the studies of DNA oxidation in autoimmune disease are confined to RA and SLE, with analysis being conducted predominantly in peripheral blood mononuclear cells (PBMC), with some analysis of urinary 8-OH-dG levels [11, 12]. It is presently difficult to assign exact roles for DNA oxidation in the pathogenesis of these conditions, largely because, beyond using lesion analysis as a biomarker of oxidative stress, studies examining functional impacts of these lesions on immune cell function or to explain damage to affected organ systems are lacking. Those studies that have measured levels of 8-OH-dG, show elevated levels of this lesion in PBMC from patients with RA and SLE (also Behcet's disease and vasculitis), plus depending on the particular study, either very low or unchanged urinary excretion of 8-OH-dG in SLE and elevated excretion of 8-OH-dG in RA, compared to healthy subjects. Elevated excretion has been taken as indicative of increased oxidative stress in RA while the reports of very low levels of 8-OH-dG in urine from SLE patients have been taken as evidence of abnormal repair of 8-OH-dG, rather than a low level of oxidative damage. Despite this, functional measurements of specific enzyme activities for the repair of oxidative DNA damage in SLE seem to be minimal. Although not specific for oxidative stress, elevated levels of DNA strand breaks, in PBMC from RA patients have also been reported, again the functional significance of this is unclear.

In the case of RA, the hyperplastic tissue in the synovial joint, the pannus, is subject to an oxidative burden which is most likely accompanied by oxidative DNA damage. Indeed the ability of cells in the pannus to deal with DNA damage may be compromised, with evidence of somatic mutations in p53 (transition mutations characteristic of oxidative damage) and potential abnormalities in mismatch repair, the latter accompanied by elevated levels of microsatellite instability. Thus, cell populations in the pannus are presented with opportunities to evade DNA repair and for mutations to be expressed. Collectively, there are several consequences of this for certain cell populations, including increased or decreased cell death, acquisition of growth advantages, or enhancement of a pro-inflammatory environment, since p53 is reported to be involved in controlling the expression of IL-6 for example. The recent demonstration that oxidatively-modified mitochondrial DNA (mtDNA) has pro-inflammatory properties,

and can induce arthritis in a mouse model, coupled with the presence of mtDNA and 8-OH-dG in synovial fluids of RA patients, implies that this material could help perpetuate a pro-inflammatory environment in the RA joint [13].

Modification of the known autoantigen, the ribonucleoprotein Ro60, in SLE by the lipid peroxidation end product 4-hydroxy-nonenal to enable neo-antigen formation and facilitate epitope spreading, links oxidative stress in this condition directly with the pathology. This apparently intimate involvement of oxidative stress with the pathology in SLE is supported further by reports of mice models, where antioxidants can significantly suppress mortality in the NZB × NZW F_1 lupus-prone mouse and a recent report implicating oxidative stress as an early event in the development of lupus-like symptoms in the Nrf2-deficient mouse (Nrf2 is a key transcription factor involved in cellular response to oxidative stress). Oxidative modification of DNA has been shown to render it more immunogenic and also markedly increase its antigenicity for anti-dsDNA antibodies characteristic of SLE [14]. This may provide a mechanism for enhancement of selected autoantigen production which, coupled with reports of elevated apoptosis and/or poor scavenging of apoptotic debris and enhanced oxidative stress in SLE perhaps leading to enhanced cell turnover, provides a route to expose the immune system to oxidatively-modified nucleic acid. Autoantibodies specifically recognising 8-OH-dG, and other lesions (5-hydroxymethyl-2′-deoxyuridine), have been isolated from SLE patients, providing support that oxidatively modified DNA may play a role in this disease [15]. Despite this evidence, the question still remains whether damage is introduced into DNA before or after cell death, either route may be possible in light of suggestions of DNA repair abnormalities in SLE; a direct demonstration of a repair deficit for oxidative DNA damage in SLE-derived neutrophils has been reported.

Neurological Disease

Oxidatively damaged DNA has been measured in several neurodegenerative conditions, most notably Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Friedreich's ataxia (FA), and Huntington's disease (HD) [16, 17]. However, whilst oxidative stress, and more particularly oxidative DNA damage, may be a feature of some neurodegenerative diseases, there is not universal agreement as to its importance. Neuronal cells would seem to be particular targets for oxidative injury because of their high metabolic activity/oxygen consumption, reliance on mitochondrial function, relatively high unsaturated lipid content and transition metal ion availability. The functional impairment and death of neuronal cells has particular importance, given the potential severity their loss can have upon numerous physiological functions such as cognition, memory, learning, motor functions

etc. A pathogenic mechanism for oxidative DNA damage in neurodegeneration is cytotoxicity, especially depletion of selected populations of neuronal cells, e.g. in the substantia nigra of Parkinsonian brains. It is this selectivity, and subsequent manifestation of symptoms, that is the intriguing aspect of oxidative damage in the CNS. The importance of mitochondria to the viability of neuronal cells makes the genome of these organelles a particularly crucial target for ROS, and is a recurrent theme in the pathogenesis of several major neurological disorders. Oxidative damage to mtDNA, leading to mutation or deletion events, the impairment of ATP production and increased ROS generation can have direct implications for damage to, and repair of, nuclear DNA (nDNA), in addition to the pivotal role of mitochondria in apoptosis. The validity of these generic concepts is reinforced by an examination of oxidative DNA damage analysis in selected neurodegenerative conditions:

(a) Alzheimer's disease (AD): Increased levels of oxidative damage to nDNA and mtDNA have been measured in selected regions of the brain in AD patients, as well as in PBMC. Whilst several of these studies have examined 8-OH-dG in particular, using immunostaining for example, others have shown elevated levels of various purine and pyrimidine lesions using chromatographic analyses [18]. Lower levels of extracellular 8-OH-dG in cerebrospinal fluid (CSF) from AD patients, compared to healthy subjects, coupled with elevated levels in cellular DNA, also suggest poor repair and persistence of oxidative DNA damage in AD. Damage to mitochondria in AD, and resultant neuronal dysfunction, is considered important for reasons discussed above, although it is not clear whether increased mitochondrial production of ROS and subsequent mitochondrial damage is an early or exacerbating neurodegenerative event in AD. Alterations in the expression of components of the mitochondrial electron transport chain have been observed in AD, along with mutations/deletions in the mitochondrial genome and correlations with elevated levels of 8-OH-dG. This is also interesting when taken in the context of the observation that mitochondrial hOGG1 (human 8-oxo-guanine DNA glycosylase) function may be impaired in regions of the AD brain. It would seem that initial irreversible damage could precipitate a vicious cycle of worsening mitochondrial function and subsequent loss of cellular integrity.

A recent report would indicate that the impact of oxidative DNA damage in the brain extends beyond neurodegenerative disease and into that syndrome to which we will all eventually succumb, ageing [4]. At least in the context of neuronal cells, this study suggests that the ageing brain is, perhaps not surprisingly, accompanied by alterations in gene expression. What is interesting is that there may be a specific role for oxidative DNA damage in the down-regulation of selected genes via oxidative modification to promoter regions, which, as well as being more susceptible to damage because of their high GC content are also

less likely to be repaired. What makes the promoter regions of particular genes more susceptible to damage compared to others awaits further study.

(b) Parkinson's disease (PD): Oxidative stress is associated with PD, demonstrated by increased levels of markers of protein, lipid and nucleic acid oxidation, along with deficits in antioxidant function. In particular, increased 8-OH-dG levels in cells of the substantia nigra, the region of the brain defective in PD, have been observed, as well as reports of increased levels of 8-OH-dG in CSF [19]. As with AD, defects in mitochondrial function and damage to mtDNA are apparent in PD, which could play a role in the selective destruction of cells of the substantia nigra. Dementia with Lewy bodies (DLB), is the second most common pathological subgroup of dementia after AD, sharing clinical similarities to PD and AD. Markers of oxidative stress are also elevated in this condition, including ring-opened purines and pyrimidine oxidation products, and higher levels of 8-OH-dG in the substantia nigra of DLB brains, albeit to a lesser extent than in PD.

(c) Amyotrophic lateral sclerosis (ALS; motor-neurone disease): Oxidative damage may play a role in both sporadic and familial ALS; increased levels of 8-OH-dG are found in the motor cortex from sporadic ALS brains and spinal cord from both sporadic and familial ALS. There is also significantly increased urinary excretion of 8-OH-dG in ALS, suggestive of increased oxidative stress. The selective neuronal degeneration observed in ALS may again include a pathogenic role for defective mitochondrial function. Deficits in mtDNA repair of oxidative damage have been noted particularly in large motor neurones of the lumbar spinal cord in sporadic ALS.

(d) Huntington's disease (HD) and Friedreich's Ataxia (FA): The genetic defects in HD (expansion of CAG repeats in exon 1 of the huntingtin gene) and FA (expansion of a GAA repeat in intron 1 of the *FRDA* gene, coding for the protein frataxin) are known. Expression of the aberrant proteins in both of these conditions affects mitochondrial electron transport, although at different points. Their effects could ultimately be similar however, and it would seem feasible that consequential oxidative damage to DNA plays a role in the pathology, including promoting a progressive deterioration of mitochondrial function and cell death. Frataxin is involved in the regulation of mitochondrial iron content and the accumulation of mitochondrial iron in FA could further enhance oxidative damage. There is markedly increased excretion of 8-OH-dG in FA patients, supporting increased DNA oxidation in this condition. While not all reports in the literature are supportive of a role for oxidative damage in HD, there would seem to be some defects in mitochondrial function, along with evidence of regional specificity in the distribution of oxidative DNA damage to mtDNA in the brain of patients with HD, suggesting such damage may be important [20].

Cardiovascular Disease

Oxidative DNA damage has been analysed primarily in atherosclerosis, in plaque tissue itself, in lymphocytes from patients with atherosclerosis or those at risk for the disease. Whilst many studies support elevated levels of lesions, particularly 8-OH-dG, these lesions have usually been measured in well established disease, thus roles for oxidative DNA damage as an early event in cardiovascular disease (CVD) is not particularly developed. As with the neurodegenerative conditions discussed above, an important target for oxidative DNA damage in CVD would seem to be mitochondria, with death of selected cell populations being an eventual outcome, e.g. in the case of cardiomyocyte injury and heart failure [21]. The mitochondria of ischaemic hearts display abnormalities in oxidative phosphorylation, as well as an increased prevalence of the common 5 kbp deletion in the mitochondrial genome. This deletion also appears more frequently in cells of the atherosclerotic plaque and even in PBMC. It has been suggested that oxidative stress may contribute to this, and other, mtDNA deletion events and its occurrence in PBMC suggests a more global oxidative stress in the vasculature of these patients [22]. The common mtDNA deletion also accumulates with age in normal hearts, suggesting deficits in mitochondrial function accompany the ageing vasculature (as well as the ageing CNS). In atherosclerosis, increased levels of oxidised DNA lesions may serve as markers of increased oxidative stress in plaques, but what function may DNA oxidation have in the development of the plaque? Mitochondrial damage and its influence on nDNA may be one pathological role, via induction of apoptosis or necrosis, for example, perhaps enabling an accumulation of cellular debris, enhancement of inflammatory cell influx, the formation of fibrotic tissue and plaque instability/rupture. Interestingly, some suggestions from the literature would indicate that carcinogenesis and atherosclerosis may share common molecular processes, with a potential pathogenic role for DNA damage [23]. In this case, this raises the possibility of (oxidative) DNA damage inducing mutation, conferring survival/growth advantages and assisting the clonal expansion of selected vascular smooth muscle cells, for example [24]. This latter area and that of the examination of somatic mutations in CVD, is receiving notable research attention, not to the detriment of the widely accepted inflammation hypothesis of atherosclerosis, but as a complement to this. Oxidative damage to DNA in the pathogenesis of cardiovascular disease evidently extends beyond heart failure and atherosclerosis, having implications for the pathogenesis of stroke, ischaemia-reperfusion injury and pre-eclampsia.

Diabetes

In both type 1 and type 2 diabetes there are many reports of elevated oxidative stress, including markers of DNA oxidation (predominantly 8-OH-dG, but

some studies have measured oxidised pyrimidines and ring-opened purines), in both extracellular matrices, peripheral blood cells and in rodent models of diabetes. As with many other studies, these lesions have been used primarily as markers of oxidative stress. There are close mechanistic links between hyperglycaemia, the oxidative stress seen in this disease and, by implication, oxidative DNA damage. The latter is thought to be involved in the development of several of the important complications of diabetes, e.g. peripheral vascular disease, cardiomyopathy, nephropathy, and renal pathology [25]. The most likely effect of this oxidative DNA damage is a role in induction of cell death, and perhaps negative effects upon gene expression. Of direct relevance to the production of insulin, oxidative DNA damage probably plays a role in the destruction of insulin-secreting pancreatic islet β-cells in both type 1 and type 2 diabetes. Several reports do again imply an important role for mitochondrial damage, not only to the metabolically active β-cells, but also to the tissues integral to the manifestation of diabetic complications, e.g. there are significant correlations between tissue levels of 8-OH-dG and ~5 kbp deletions in mtDNA from muscle of patients with non-insulin dependent diabetes mellitus and in renal tissues of diabetic rats [26].

Conclusions: DNA Oxidation – Biomarker, Biohazard . . . or Both?

A pathogenic role for oxidatively-damaged DNA in non-malignant disease is perhaps, for some, difficult to conceive. In part this is because in many conditions where oxidative DNA damage has been measured, little consideration has been given to pathogenic roles for such damage. It is perfectly valid to use measures of oxidative DNA damage as markers of on-going oxidative stress, although the utility of such measurements would be strengthened by parallel assessment of other markers such as indices of protein/lipid oxidation or assessment of antioxidant status. The relative importance of DNA oxidation in disease pathogenesis is another area worthy of consideration. The measurement of DNA oxidation products in various disease states has outstripped work on their pathogenic significance, as has the, perhaps understandable, pre-occupation with 8-OH-dG. This pre-occupation probably results from the quantity and quality of the information (8-OH-dG is the subject of most of the reports measuring oxidative DNA damage and is regarded as a biologically important lesion) passed on to the wider medical research community from those laboratories concerned with the measurement of DNA oxidation. Compared to 8-OH-dG, there have been relatively few studies examining other products of DNA oxidation, in terms of their measurement in disease and biological impact, and redressing the balance would be welcome.

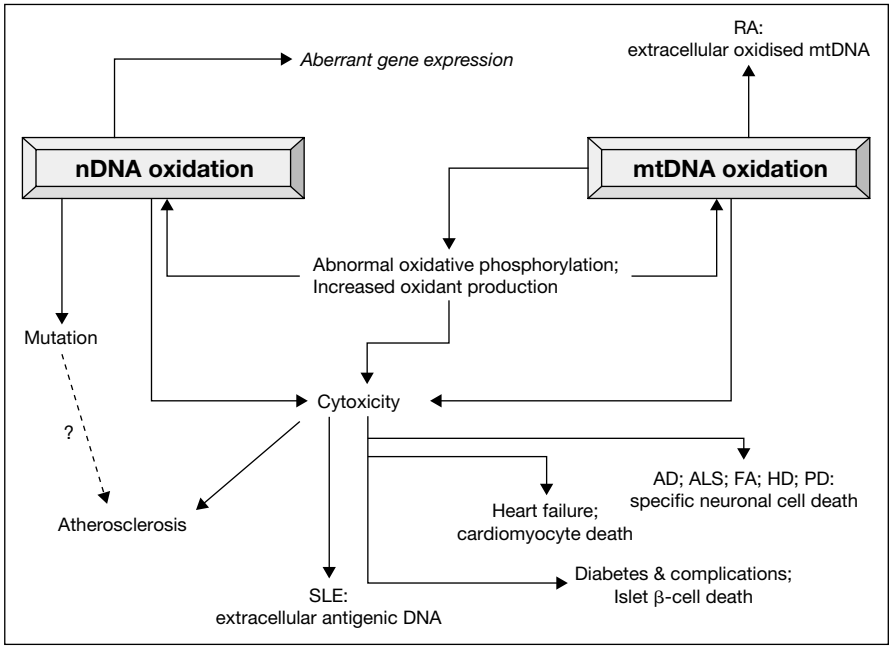

Fig. 2. Summary of pathological impacts of oxidative damage to DNA in non-malignant conditions, with references to specific evidence in particular disease states. Oxidant-mediated alteration in gene expression would have numerous impacts on cell function and survival and thus may overarch the other phenomena in the figure. The role of somatic mutation in atherosclerosis is still tentative. AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; FA: Friedreich's ataxia; HD: Huntington's disease; mtDNA: mitochondrial DNA; nDNA: nuclear DNA; PD: Parkinson's disease; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus.

Evidently, there is greater added value to the assessment of markers of oxidative DNA damage if pathogenic roles can be ascribed to this damage. Although some laboratories have gone beyond simply measuring oxidative DNA damage, many studies do not follow-up with experimental investigations as to why such damage may be important in disease, leaving conclusions to be pure conjecture by the authors. This is an area that is undergoing some expansion and we see this as necessary in order for the field to catch up with the plethora of reports using oxidative DNA damage simply as a biomarker. Figure 2 summarises potential pathological roles of oxidative DNA damage in the various disease states mentioned in this chapter.

What is evident to us, is that nuclear DNA is not necessarily the most important target genome, and we also suggest that a viewpoint solely linking

oxidative DNA damage with mutation is perhaps naive. The effects of oxidative DNA damage on epigenetic phenomena, and the control of gene expression is, we feel, set to develop over the coming years and provide renewed impetus and relevance to the free radical field. At this stage, our conclusion is perhaps predictable: oxidative DNA damage in non-malignant conditions can represent both a biohazard and a biomarker. However the extent to which the damage extends beyond being a biomarker to play a role in the pathology, and also the timing of damage in relation to the pathogenesis of the disease, also appears to depend on the condition studied.

References

1 Halliwell B, Gutteridge JMC: Free Radical Biology and Medicine, 3rd ed. Oxford University Press, Oxford, 1999.
2 Evans MD, Dizdaroglu M, Cooke MS: Oxidative DNA damage and disease: induction, repair and significance. Mutat Res 2004;567:1–61.
3 Evans MD, Cooke MS: Factors contributing to the outcome of oxidative damage to nucleic acids. BioEssays 2004;26:533–542.
4 Lu T, Pan Y, Kao S-Y, Li C, Kohane I, Chan C, Yankner BA: Gene regulation and DNA damage in the ageing human brain. Nature 2004;429:883–891.
5 ESCODD (European Standards Committee on Oxidative DNA Damage): Comparative analysis of baseline 8-oxo-7,8-dihydroguanine in mammalian cell DNA, by different methods in different laboratories: an approach to consensus. Carcinogenesis 2002;23:2129–2133.
6 Cooke MS, Evans MD, Dove R, Rozalski R, Gackowski D, Siomek A, Lunec J, Olinski R: DNA repair is responsible for the presence of oxidatively damaged DNA lesions in urine. Mutat Res 2005;574:58–66.
7 Cooke MS, Lunec J, Evans MD: Progress in the analysis of urinary oxidative DNA damage. Free Radic Biol Med 2002;33:1601–1614.
8 Siomek A, Gackowski D, Foksinski M, Gran C, Klungland A, Olinski R: Diet is not responsible for the presence of several oxidatively damaged DNA lesions in mouse urine. Free Radic Res 2004;38:1201–1205.
9 Rogstad DK, Liu P, Burdzy A, Lin SS, Sowers LC: Endogenous DNA lesions can inhibit the binding of the AP-1 (c-Jun) transcription factor. Biochemistry 2002;41:8093–8102.
10 Kang D, Hamasaki N: Alterations of mitochondrial DNA in common diseases and disease states: aging, neurodegeneration, heart failure, diabetes, and cancer. Curr Med Chem 2005;12:129–441.
11 Bashir S, Harris G, Denman MA, Blake DR, Winyard PG: Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. Ann Rheum Dis 1993;52:659–666.
12 Evans MD, Cooke MS, Akil M, Samanta A, Lunec J: Aberrant processing of oxidative DNA damage in systemic lupus erythematosus. Biochem Biophys Res Commun 2000;273:894–898.
13 Collins LV, Hajizadeh S, Holme E, Jonsson IM, Tarkowski A: Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses. J Leukoc Biol 2004;75:995–1000.
14 Cooke MS, Mistry N, Wood C, Herbert KE, Lunec J: Immunogenicity of DNA damaged by reactive oxygen species – implications for anti-DNA antibodies in lupus. Free Radic Biol Med 1997;22:151–159.
15 Frenkel K, Karkoszka J, Kim E, Taioli E: Recognition of oxidized DNA bases by sera of patients with inflammatory diseases. Free Radic Biol Med 1993;14:483–494.
16 Cooke MS: Neurodegenerative disease and the repair of oxidatively damaged DNA; in Beal MF, Lang AE, Ludolph A (eds): Neurodegenerative Diseases. Cambridge University Press, Cambridge, 2005.
17 Sayre LM, Smith MA, Perry G: Chemistry and biochemistry of oxidative stress in neurodegenerative disease. Curr Med Chem 2001;8:721–738.

18 Migliore L, Fontana I, Colognato R, Coppede F, Siciliano G, Murri L: Searching for the role and the most suitable biomarkers of oxidative stress in Alzheimer's disease and in other neurodegenerative diseases. Neurobiol Aging 2005;26:587–595.
19 Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B: Oxidative DNA damage in the Parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J Neurochem 1997;69:1196–1203.
20 Schapira AH: Mitochondrial involvement in Parkinson's disease, Huntington's disease, hereditary spastic paraplegia and Friedreich's ataxia. Biochem Biophys Acta 1999;1410:159–170.
21 Tsutsui H: Oxidative stress in heart failure: the role of mitochondria. Intern Med 2001;12: 1177–1182.
22 Botto N, Berti S, Manfredi S, Al-Jabri A, Federici C, Clerico A, Ciofini E, Biagini A, Andreassi MG: Detection of mtDNA with 4977 bp deletion in blood cells and atherosclerotic lesions of patients with coronary artery disease. Mutat Res 2005;570:81–88.
23 Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS: Atherosclerosis and cancer: common molecular pathways of disease development and progression. Ann NY Acad Sci 2001;947: 271–293.
24 Andreassi MG, Botto N: Genetic instability, DNA damage and atherosclerosis. Cell Cycle 2003;2: 224–227.
25 Hinokio Y, Suzuki S, Hirai M, Chiba M, Hirai A, Toyota T: Oxidative DNA damage in diabetes mellitus: its association with diabetic complications. Diabetologia 1999;42:995–998.
26 Suzuki S, Hinokio Y, Komatu K, Ohtomo M, Onoda M, Hirai S, Hirai M, Hirai A, Chiba M, Kasuga S, Akai H, Toyota T: Oxidative damage to mitochondrial DNA and its relationship to diabetic complications. Diabetes Res Clin Pract 1999;45:161–168.

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Dominant Non-Coding Repeat Expansions in Human Disease

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Abstract

The general model that dominant diseases are caused by mutations that result in a gain or change in function of the corresponding protein was challenged by the discovery that the myotonic dystrophy type 1 mutation is a CTG expansion located in the 3′ untranslated portion of a kinase gene. The subsequent discovery that a similar transcribed but untranslated CCTG expansion in an intron causes the same multisystemic features in myotonic dystrophy type 2 (DM2), along with other developments in the DM1 field, demonstrate a mechanism in which these expansion mutations cause disease through a gain of function mechanism triggered by the accumulation of transcripts containing CUG or CCUG repeat expansions. A similar RNA gain of function mechanism has also been implicated in fragile X tremor ataxia syndrome (FXTAS) and may play a role in pathogenesis of other non-coding repeat expansion diseases, including spinocerebellar ataxia type 8 (SCA8), SCA10, SCA12 and Huntington disease-like 2.

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Fragile X mental retardation (FMR) and Friedreich's ataxia (FA), both caused by non-coding triplet repeat expansions, were among the first trinucleotide repeat disorders described [1, 2]. Consistent with the paradigm that recessive disorders are caused by loss of function mechanisms, these non-coding expansions prevent normal protein expression [1, 2]. The discovery that dominant diseases such as Huntington disease and several spinocerebellar ataxias are caused by CAG expansions that are translated into polyglutamine tracks affecting their corresponding proteins, follows the paradigm that dominant diseases are caused by a gain or change of function of the corresponding mutant protein [3]. This straightforward and relatively simple model

was complicated by the discovery that dominantly inherited myotonic dystrophy type 1 (DM1) is caused by an untranslated CTG expansion in the 3′ noncoding region of the *DMPK* gene [4–6]. The discovery that a second form of myotonic dystrophy, myotonic dystrophy type 2 (DM2), is caused by a noncoding CCTG repeat expansion, provided strong support for a gain of function mechanism in which microsatellite expansions can be pathogenic at the RNA level [7]. There is now a growing body of evidence that repeat expansions expressed at the RNA level, but not the protein level, cause fragile-X tremor ataxia syndrome (FXTAS) [8, 9] and possibly other disorders including spinocerebellar ataxia type 8 (SCA8), SCA10, SCA12, and Huntington's disease like 2 (HDL2) [10–13].

Myotonic Dystrophy Type 1: Identification of the First Dominant Non-Coding Expansion Disorder

In 1992, a CTG expansion in the 3′ untranslated region of the dystrophia myotonica-protein kinase gene (*DMPK*) was shown to cause myotonic dystrophy (DM), the most common form of muscular dystrophy in adults [4, 5]. For the following seven years this was the only example of an untranslated trinucleotide expansion causing a dominant disease; the mechanism underlying the pathogenesis of this unusual mutation remained elusive because the dominant inheritance pattern was difficult to reconcile with the location of the expansion in a portion of the kinase gene that would not affect the protein sequence [14]. Several models to explain how the CTG expansion might cause the unusual constellation of features associated with DM1 included: (i) haploinsufficiency of the DMPK protein; (ii) altered expression of neighboring genes, including the homeodomain gene *SIX5*; and (iii) pathogenic effects of the CUG expansion in RNA, which accumulates as ribonuclear foci in affected tissue and causes downstream disruptions in alternative splicing [10, 14]. The mouse models created to elucidate the pathogenesis for DM1 each developed aspects of the multisystemic phenotype: an RNA gain of function model that Mankodi et al. developed, in which a CTG expansion of ~250 repeats was expressed at the 3′ UTR of the unrelated human skeletal actin gene causes myotonia and myopathic features [15]; a homozygous *Dmpk* knockout develops cardiac abnormalities [16, 17]; and a knockout model of the neighboring *Six5* gene causes cataracts, though not the specific posterior iridescent cataracts seen in patients [18, 19]. Taken together, each of these transgenic mice was thought to provide support for the concept that DM1 is a regional gene disorder with each of the proposed mechanisms contributing to disease pathogenesis [14]. While the additive model was appealing, an inconsistency was that the genetic locus for a

second form of myotonic dystrophy (DM2) had been mapped to a different chromosome. The lack of synteny between the DM2 region on chromosome 3 with the DM1 locus on chromosome 19 made a mechanism involving the additive effects of multiple dysregulated genes at the DM1 locus seem unlikely [14, 20].

Discovery of DM2 Sheds Light on the Pathogenicity of Non-Coding Repeats

After genetic testing became available for DM1 in 1992, some individuals with the rare and complex clinical presentation of DM were identified who did not have the CTG expansion [21, 22]. As in DM1, these patients had myotonia, characteristic histological abnormalities in skeletal muscle, posterior iridescent cataracts, severe cardiac arrhythmias, hypotestosteronism and oligospermia, and insulin insensitivity [23]. We identified and subsequently used a large family with similar multisystemic clinical features to map the second locus, designated myotonic dystrophy type 2 (DM2), to chromosome 3q21 [20, 24].

In 2001, Liquori et al. demonstrated that DM2 is caused by a CCTG repeat expansion in intron 1 of the zinc finger protein 9 (*ZNF9*) gene. Similar to DM1, the DM2 repeat tract is transcribed but not translated [7] and CCUG-containing ribonuclear inclusions accumulate in DM2 skeletal muscle [7]. Although DM2 patients often have a milder disease course than individuals with DM1, the DM2 CCTG repeat expansions are generally much larger than those for DM1, ranging in size from ~75 to 11,000 repeats with a mean of ~5,000 repeats [25]. Because somatic instability causes a broad range of DM2 allele sizes in individual patients, which are evident as smears by Southern analysis – rare individuals with the smallest repeat expansions (75–100 CCTGs) also have other larger expansion sizes, therefore preventing a clear definition of the smallest pathogenic repeat size [7].

The function of *ZNF9* and surrounding genes at the DM2 locus (*KIAA1160*, *Rab11B*, glycoprotein IX, *FLJ11631*, and *FLJ12057*) bear no obvious similarity to the genes found at the DM1 locus (*SIX5* and *DMWD*) [14, 26, 27]. Although the additive model of DM1 proposed that RNA containing the CUG expansion caused myotonia and muscular dystrophy, it was also thought that the haploinsufficiency of *DMPK* and surrounding genes such as *SIX5* contributed to other features of the disease, including cardiac conduction defects and cataracts [25]. The isolation of the DM2 mutation and the clinical and molecular parallels between DM1 and DM2 now provide strong support for the model in which the broad constellation of clinical features associated

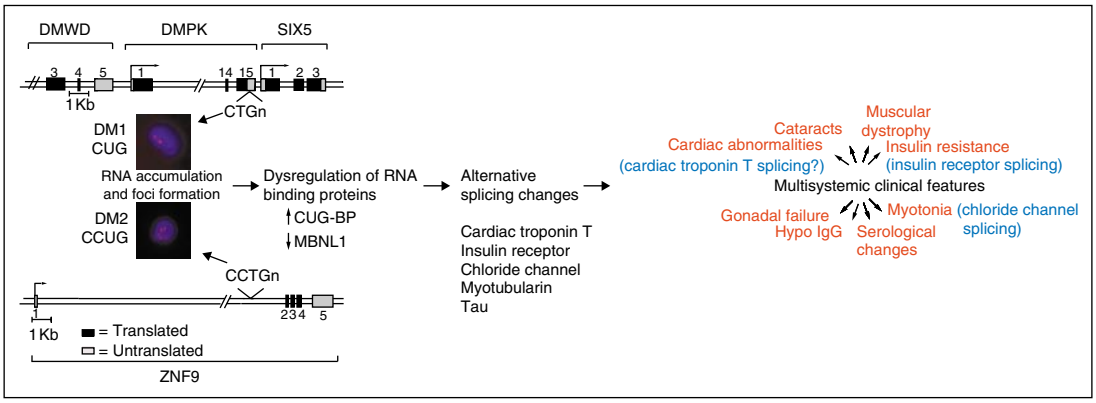

Fig. 1. RNA model of DM pathogenesis. Clinical and molecular parallels between DM1 and DM2 simplify previous models of DM pathogenesis and demonstrate that the multisystemic features shared by DM1 and DM2 are caused by an RNA mechanism mediated by CUG- and CCUG-containing transcripts. CUG- and CCUG-containing transcripts accumulate as ribonuclear foci. The subsequent dysregulation of RNA binding proteins, including CUG-BP and the muscleblind protein MBNL1, leads to disruptions in the regulation of normal alternative splicing, leading to the multisystemic features common to DM1 and DM2 ([86], reprinted with permission from Elsevier©).

with DM1 and DM2 is caused by a gain of function RNA mechanism (fig. 1) [7, 28].

Evidence Supporting an RNA Gain of Function Mechanism in the Myotonic Dystrophies

The following lines of evidence support an RNA gain of function model for the myotonic dystrophies: a) the DM1 expansion in the 3′UTR of the *DMPK* mRNA has the ability to inhibit myoblast differentiation in cell culture and myogenesis in murine models [29, 30]; b) repeat tracts of more than 250 CTGs cause myotonia and myopathy when expressed at the RNA level in the 3′ UTR of the *DMPK* or human skeletal muscle actin (*HSA*) gene in transgenic animals [15, 31]; c) specific RNA binding proteins, CUG-BP and three forms of muscleblind (MBNL, MBLL, and MBXL), have altered regulation or localization in cell culture models of both DM1 and DM2 [32–34]. The RNA transcripts containing expanded CUG repeat tracks cause an increase in CUG-BP protein, however, the mechanism for this increase is currently unknown [35, 36]. In contrast, expanded DM1 and DM2 transcripts are thought to

decrease available muscleblind protein, possibly by its sequestration in the repeat containing ribonuclear inclusions [34, 37]. Alterations in CUG-BP and MBNL expression levels subsequently induce disease-related splicing changes, including alterations of skeletal muscle chloride channel (*ClC-1*) and insulin receptor (*IR*) transcripts, which are in turn thought to cause the DM associated myotonia and insulin resistance, respectively [36, 38–40]. Recent experiments in transfected cells have suggested that CUG expansions can sequester transcription factors, causing downstream dysregulation of gene expression, although this effect has not yet been demonstrated in DM tissue [41]. The various effects that the DM1 and DM2 repeat expansions have on specific molecules are discussed in detail below.

The Role of RNA Binding Proteins: CUG-BP and Muscleblind (MBNL)

Initial efforts to investigate an RNA gain of function mechanism were focused on identifying proteins that could bind to the DM1 CUG repeat motif. In 1996, Timchenko et al. identified CUG-binding protein (CUG-BP), a member of the CELF family of proteins that regulate pre-mRNA alternative splicing [32, 42]. They demonstrated that phosphorylation and localization of CUG-BP is altered in DM1 cells [43]. In 2004, Timchenko et al. reported that overexpression of CUG-BP1 in the skeletal muscle of mice causes muscular dystrophy, altered myofiber type, and inhibition of myogenesis [44]. A second mouse model, in which CUG-BP1 is overexpressed in heart and skeletal muscle using a muscle-specific promoter, results in neonatal lethality and classic DM associated changes – chains of central nuclei in skeletal muscle fibers, degenerating muscle fibers and typical splicing abnormalities of *Clcn1*, *Mtmr1*, and *Tnnt2* transcripts [45].

In contrast to CUG-BP, the MBNL proteins co-localize with the CUG and CCUG foci, initially suggesting that this protein may be linked to DM pathogenesis [33, 34, 46–49]. The significant role of muscleblind in DM pathogenesis was demonstrated by Kanadia et al., in a *Mbnl1* knockout mouse model ($Mbnl1^{\Delta E3/\Delta E3}$) in which the mice develop myotonia, cataracts, and splicing alterations reminiscent of DM [37]. Data currently indicate that either depletion of MBNL or overexpression of CUG-BP can recapitulate features of myotonic dystrophy [37, 44, 45].

CUGBP and MBNL Mediate the Dysregulation of Alternative Splicing

Characteristic of both DM1 and DM2 are changes in the regulation of alternative splicing of a specific set of transcripts, including cardiac troponin T (*cTNT*),

the insulin receptor (*IR*), muscle specific chloride channel (*ClC-1*), myotubularin-related 1 (*MTMR1*), and microtubule-associated protein tau (*MAPT*) [36, 38–40, 50–53]. To investigate the molecular mechanisms underlying these alternative splicing changes, cell culture and animal models of CUG-BP and MBNL have been developed and are discussed below [35–40, 44, 45, 54].

Cardiac Troponin T (cTNT). In 1998, Philips et al. established that CUG-BP mediated the dysregulation of alternative splicing of the cardiac troponin T (*cTNT*) gene through its altered interaction with CUG expansion containing transcripts [54]. This was the first demonstration that the expanded CUG repeat tract could have a *trans*-dominant effect on splicing of another gene. In 2005, Ho et al. extended this analysis and reported that splicing of the *cTNT* gene is altered in the heart of mice overexpressing *CUG-BP1* [45]. Similarly, cardiac tissue from the *Mbnl* knockout model and DM patients show a predominant expression of the fetal isoform of cardiac troponin T containing exon 5. Similar splicing changes of the fast skeletal muscle troponin T are also disrupted in this mouse model with similar findings in human DM1 muscle [37]. Ladd et al. have recently shown that the developmental regulation of *cTNT* alternative splicing is determined by a balance of positive (CUG-BP) and negative (MBNL) regulation. When the expression of these regulatory proteins is altered, as is the case in DM, there is aberrant splicing of the target genes [55].

Muscle-Specific Chloride Channel (ClC-1) Gene. A reduction in the level of transmembrane chloride conductance is capable of causing myotonia, a classic feature of both DM1 and DM2, in which voluntary muscle contraction is followed by involuntary repetitive firing of action potentials that prevent muscle relaxation [15, 38, 56]. Mankodi et al. used a transgenic mouse model of DM1 to demonstrate that the expression of CUG repeat expansions causes a reduction in sarcolemmal chloride conductance to levels associated with myotonia [15, 38]. Further examination of mouse models and both DM1 and DM2 patients revealed aberrant splicing of the muscle-specific chloride channel (*ClC-1* in humans or *Clcn1* in mice), which results in a loss of the channel at the plasma membrane [38, 39]. CUG-BP, which is elevated in DM1, binds to the *ClC-1* pre-mRNA and causes aberrant splicing of the transcript. Overexpression studies of *CUG-BP* in cell culture and mice recapitulate the abnormal *ClC-1* splice patterns characteristic of DM [39, 45]. Similar splicing alterations also occur in the *Mbnl* knockout mice, indicating that the splicing is regulated antagonistically – increased activity of CUGBP1 or decreased activity MBNL causes the dysregulation of alternative splicing that often favors the expression of fetal splice forms [37, 45]. These studies support a model in

which aberrant splicing of the *ClC-1* mRNA can lead to the channelopathy and membrane hyperexcitability that underlie the myotonia that is classically associated with DM [37–39, 45].

Insulin Receptor (IR). Both DM1 and DM2 patients classically present with insulin resistance, a precursor to diabetes [10]. This reduced response to insulin in the skeletal muscle results from aberrant splicing of the insulin receptor (*IR*) pre-mRNA. An insulin insensitive splice form that lacks exon 11 is the predominant transcript in muscle from affected patients as well as cell culture models of DM because CUG-BP binds to a splicing enhancer located upstream of exon 11 [36, 40]. These results support the model that the increased *CUG-BP* expression in DM enhances expression of the insulin insensitive splice form that results in the characteristic insulin resistance [36, 40].

Splicing Alterations Associated with DM1: Aberrant Expression of Tau and Myotubularin Transcripts. In both DM1 patients and a mouse model by Seznec et al., aberrant splicing of the microtubule-associated protein tau pre-mRNA has been observed in CNS tissue [31, 53, 57]. Additionally, the myotubularin-related 1 (*MTMR1*) gene shows muscle-specific alterations in the normal splice pattern in cultured muscle cells and skeletal muscle samples from patients with congenital onset DM1 [51] and in mice overexpressing *CUG-BP1* [45]. These data suggest that the overexpression of fetal isoforms and a delayed switch to adult *Mtmr1* splice forms may play a role in the severe hypotrophy of congenital DM1 muscle [51].

Other Potential Targets of CELF Protein Splicing. Recently, Faustino and Cooper reported new putative splicing targets for CUGBP2 (ERT-3), another member of the CELF family of proteins involved in the regulation of splicing [58]. Using systematic evolution of ligands by exponential enrichment (SELEX), the preferred binding site of CUGBP2 was identified along with putative targets, including *Ros1*, *BITE*, *MEF2C*, *MTMR1*, *MYH7*, *FXRP1*, *AGA*, *MBNL*, *NCX1*, *CFTR*, *FREQ*, *TPM1*, *FLJ12871*, *GRID2*, *FOXM1*, *VCL*, *RASGRF1* and *ATP2A2*. It is possible that transcripts of some of these genes may also be alternatively spliced in DM, but further experimentation is required.

Taken together, these data predict that the splicing changes characteristic of DM would result from overexpression of either the CUG/CCUG repeat expansion or CUG-BP or the depletion of MBNL [35–37, 40, 44, 45, 55].

Potential Molecular Mechanisms Contributing to the Clinical Distinctions between DM1 and DM2

While DM1 and DM2 exhibit a strikingly similar clinical presentation, DM2 is generally milder without the reported congenital form or mental retardation that can be associated with DM1 [28]. While it is now largely accepted that a gain of function RNA mechanism is likely to cause the common clinical features of these diseases, it has been proposed that the clinical differences between DM1 and DM2 may be caused by potential differences in the affinities of RNA binding proteins for the CUG vs. CCUG repeat motifs. Alternatively, changes in the expression of locus specific genes could account for the clinical distinctions between the two forms of DM. Additionally, although *DMPK* and *ZNF9* are both broadly expressed, differences in their spatial and temporal expression patterns could account for the clinical distinctions between the two diseases [10]. Filippova et al. have demonstrated that methylation at the DM1 locus in congenital cases can increase *DMPK* expression, suggesting that increased expression levels of CUG containing transcripts may cause the more severe phenotype [59].

Fragile-X-Associated Tremor/Ataxia Syndrome (FXTAS) and a Possible RNA Gain-of-Function Mechanism

Three clinically distinct diseases are associated with mutations in the *FMR1* gene: fragile-X syndrome, premature ovarian failure (POF), and fragile X-associated tremor/ataxia syndrome (FXTAS) [8]. CGG repeat expansions within the *FMR1* gene cause fragile-X syndrome if the expansion is greater than 200 repeats, however, smaller premutations (55–200 CGGs) are associated with POF and FXTAS. The large fragile-X associated expansions prevent transcription and translation of the *FMR1* gene, supporting a loss-of-function mechanism for fragile X syndrome. However, increased mRNA levels of *FMR1* have been reported for POF and FXTAS, suggesting that pathogenicity of the CGG premutations may involve an RNA gain-of-function mechanism [8, 9].

Characterizing the Clinical and Molecular Features of FXTAS

Clinical evaluations of male premutation carriers (ranging in age from 50–70) identified FXTAS, which is characterized by progressive intention tremor and gait instability, with variable memory and executive-function deficits and gradual cognitive decline [9, 60]. MRI analysis shows global brain atrophy and white matter abnormalities; while neurohistological studies of

autopsy tissue from elderly premutation carriers demonstrates cerebellar degeneration. Further, neuronal and astroglial eosinophilic ubiquitin-positive intranuclear inclusions were also apparent, suggesting the involvement of the proteosome degradation pathway that has also been implicated in the polyglutamine disorders [9].

FXTAS Fly and Mouse Models

To further elucidate the underlying molecular mechanisms of FXTAS and to test the RNA gain of function hypothesis, both mouse and fly models have been developed. A premutation repeat track of approximately 100 CGGs was knocked into the mouse *Fmr1* gene, replacing the normal repeat [61]. These mice did not develop a neurological phenotype (up to 72 weeks) but neuropathology showed a broad distribution of ubiquitin-positive intranuclear inclusions, analogous to those found in FXTAS patients. Inclusion formation occurred without an increase in FMR protein, thereby supporting the proposal that either the CGG expansion itself or elevated levels of *FMR1* mRNA are responsible for inclusion development [61]. Further characterization of this murine model has demonstrated that these mice undergo an age-dependent cognitive and neuromotor decline which may correspond to the progressive cognitive and behavioral impairment associated with FXTAS [62].

Premutation length CGG repeats expressed in the 5′ UTR of the *FMR1* gene in a *Drosophila* model cause a neurodegenerative eye phenotype that is dosage and repeat length dependent – demonstrating that CGG repeats alone can cause neurodegeneration [63]. Consistent with the neuropathological studies of human and mouse tissue, these flies also develop ubiquitin positive inclusions, even though no mutant protein is produced. Interestingly, the FXTAS inclusions also contain Hsp70, which suppresses the neurodegeneration caused by the CGG containing transcripts in other neurodegenerative models [63].

FXTAS: Evidence for an RNA Gain-Of-Function Mechanism

Data derived from patients and the fly and mouse models indicate that expression of transcripts containing premutation size CGG repeat tracts cause neurodegeneration and the formation of nuclear inclusions reminiscent of the inclusions found in polyglutamine disorders [8, 9, 63]. Because the mutation is not translated into a mutant protein it appears that the expanded CGG triggers the formation of inclusions that then recruit other proteins [8, 9, 63]. Further characterization of the inclusions and whether or not they are pathogenic or protective should provide additional insight into the disease mechanism. Whether FXTAS shares additional molecular features common to other RNA expansion disorders, such as alterations in alternative splicing, remains to be determined.

A Second Dominantly Inherited CTG Expansion Disorder – Spinocerebellar Ataxia Type 8

SCA8 is a slowly progressive, relatively pure cerebellar disease, characterized by gait and limb ataxia, nystagmus, and dysarthria [64, 65]. In 1999, we used RAPID cloning to directly isolate the SCA8 CTG expansion using DNA from a single ataxia patient [64, 66]. The SCA8 expansion, which is located in the 3′ end of a presumably processed, non-coding mRNA, was subsequently identified in other ataxia families, including a seven generation family (MN-A) [64, 65]. The *SCA8* gene overlaps the 5′ end of Kelch-like 1 (*KLHL1*), a gene encoding an actin binding protein that is transcribed in the opposite direction [64, 67, 68].

SCA8 is transmitted in an autosomal dominant pattern with reduced penetrance and in the MN-A family has a pathogenic threshold of ~110 combined repeats [64]. Although there is a tight correlation between repeat size and disease in the MN-A family this trend is not observed in most of the other SCA8 families that have been reported. The inheritance pattern in these families is complex with only a subset of expansion carriers developing the disease [64, 65, 69–71]. Since RAPID cloning eliminates the genetic biases inherent in traditional positional cloning methods, and is not dependent on large pedigrees or highly penetrant inheritance patterns, the significantly reduced penetrance that characterizes SCA8 is not surprising. Multiple factors may influence SCA8 disease penetrance including sequence interruptions of the CTG expansion and the size of the CTA tract that precedes the CTG expansion [64, 72, 73].

To further elucidate the molecular mechanisms underlying SCA8 pathogenesis, we developed a transgenic mouse model using human BAC clones that contain either 11 or 118 CTG repeats [74]. The BAC expansion animals but not the BAC control lines develop a progressive neurological phenotype that is lethal in higher copy number, indicating that expression of *SCA8* transcripts containing an expanded CTG repeat is pathogenic [74]. Additionally, Mutsuddi et al. developed a *Drosophila* model in which retinal expression of *SCA8* transcripts with both normal and expanded repeat tracts cause progressive neurodegeneration [75]. A genetic modifier screen using this neurodegenerative phenotype as a sensitized background identified three enhancer mutations in muscleblind, split ends, staufen and a suppressor mutation in *CG3249* [75]. The interaction between SCA8 and muscleblind varies with CTG repeat size, suggesting the possibility that the SCA8 expansion can alter interactions with RNA binding proteins [75].

The molecular parallels between the DM and SCA8 mutations, combined with the known toxic properties of expanded CUG containing transcripts, suggests a similar RNA gain-of-function mechanism may be involved in SCA8 pathogenesis [10, 76]. The clear phenotypic differences between SCA8, DM1, and DM2

could result from spatial and temporal expression differences of the *SCA8*, *DMPK*, and *ZNF9* transcripts – *SCA8* is almost exclusively expressed in the CNS [64, 77] while *DMPK* and *ZNF9* have broad expression patterns [7, 78].

Spinocerebellar Ataxia Type 10 (SCA10) – RNA Gain of Function vs. Protein Loss of Function

Spinocerebellar ataxia type 10 (SCA10), a dominantly inherited ataxia characterized by seizures, polyneuropathy, pyramidal signs, cognitive and neuropsychiatric impairment [79, 80], is caused by an ATTCT repeat expansion in intron 9 of the *E46L* gene, which encodes a cytoplasmic protein (475 aa) belonging to the family of armadillo repeat proteins [80–82]. The repeat expansion that causes SCA10 is unusually large with a mean size of 4,500 repeats [81]. Based on the molecular parallels between SCA10 and the myotonic dystrophies, it has been proposed that an RNA gain of function mechanism may be involved in the disease pathogenesis [13]. As is true for many neurodegenerative disorders, the ubiquitous expression of *E46L* is much broader than the sites of CNS pathology, suggesting the involvement of secondary proteins that interact with the expansion [81]. Haploinsufficiency of *E46L* has been considered an unlikely cause of this dominantly inherited disease, since *E46L* transcripts are not obviously reduced in affected individuals [81]. Interestingly, siRNA knock-down of the *E46L* gene causes increased apoptosis in neuronal cultures suggesting the possibility that decreases in expression of *E46L* may play a role in SCA10 pathogenesis. Additional experiments will be needed to clarify the pathogenic mechanism of this interesting disease [80, 82].

Huntington Disease Like 2 (HDL2) and Spinocerebellar Ataxia Type 12 (SCA12): Coding and Non-Coding Pathogenic Expansions

Similar to Huntington's disease, HDL2 is a dominantly inherited neurodegenerative disorder that results in a movement disorder, dementia and psychiatric abnormalities [11]. HDL2 results from a CTG repeat expansion in the junctophilin-3 gene (*JP-3*), which is alternatively spliced. Expanded polyleucine or polyalanine tracts are predicted to result from two of the known splice forms and may be pathogenic. However, in two other transcripts the CTG repeat is located in a non-coding portion of the gene – in either intron 1 or as part of the 3′ UTR, suggesting that disease pathogenesis of HDL2 may also involve an RNA gain of function mechanism [11].

Spinocerebellar ataxia type 12 is a neurodegenerative disease in which ataxia, tremor, and dementia are the most prominent features [83]. SCA12 is caused by a CAG repeat expansion in the *PPP2R2B* gene, which encodes a regulatory subunit of the protein phosphatase PP2A enzyme that is specifically expressed in brain [84]. The expansion is located at the 5′ end of the gene and, depending on the transcription start site, can be located within the transcript. The molecular pathogenesis of SCA12 is currently unclear. The most straightforward model appears to be that the expansion causes increased expression of the PPP2R2B protein but other models, including the possibility that the CAG expansion is pathogenic at the RNA level, are also being considered [12, 84, 85].

Conclusion

The discovery of the DM2 mutation simplified previous models of myotonic dystrophy pathogenesis and established that the multisystemic features common to both diseases are the result of an RNA gain of function mechanism [7]. In addition to DM1 and DM2, there are a growing number of dominantly inherited microsatellite expansion diseases that also appear to be caused by non-coding repeats, including SCA8, SCA10, SCA12, HDL2, and FXTAS. While DM1 and DM2 involve an RNA gain of function mechanism, the molecular mechanisms responsible for these other disorders are potentially more complex. While CGG full-mutation expansions in the *FMR* gene are pathogenic due to a loss of function mechanism, pre-mutation expansions result in an RNA gain of function disorder, FXTAS, a dominant late onset disease affecting both males and females. In contrast to the paradigm that dominant disorders result from gain of function protein mutations, SCA8, SCA10, SCA12 and HDL2 may be caused by non-coding expansions, with a variety of potential mechanisms suggested, including RNA gain of function, loss of function and increased protein expression. The simple model that recessive disorders result from loss of normal function, and that gain of function mechanisms account for dominant disorders, has evolved into a complex paradigm involving a variety of mechanisms that will become increasingly clear as research delves further into the field of non-coding disorders.

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References

1 Jin P, Warren ST: Understanding the molecular basis of fragile X syndrome. Hum Mol Genet 2000;9:901–908.
2 Pandolfo M: Molecular genetics and pathogenesis of Friedreich ataxia. Neuromuscul Disord 1998;8:409–415.
3 Orr HT, Zoghbi HY: SCA1 molecular genetics: a history of a 13 year collaboration against glutamines. Hum Mol Genet 2001;10:2307–2311.
4 Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H, Hunter K, Stanton VP, Thirion JP, Hudson T, et al: Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. Cell 1992;69:385.
5 Fu YH, Pizzuti A, Fenwick RG Jr, King J, Rajnarayan S, Dunne PW, Dubel J, Nasser GA, Ashizawa T, de Jong P, et al: An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science 1992;255:1256–1258.
6 Groenen P, Wieringa B: Expanding complexity in myotonic dystrophy. Bioessays 1998;20:901–912.
7 Liquori C, Ricker K, Moseley ML, Jacobsen JF, Kress W, Naylor S, Day JW, Ranum LPW: Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of *ZNF9*. Science 2001;293:864–867.
8 Oostra BA, Willemsen R: A fragile balance: FMR1 expression levels. Hum Mol Genet 2003;12 Spec No 2:R249–R257.
9 Hagerman PJ, Hagerman RJ: The fragile-X premutation: a maturing perspective. Am J Hum Genet 2004;74:5.
10 Ranum LP, Day JW: Pathogenic RNA repeats: an expanding role in genetic disease. Trends Genet 2004;20:506–512.
11 Holmes SE, O'Hearn E, Rosenblatt A, Callahan C, Hwang HS, Ingersoll-Ashworth RG, Fleisher A, Stevanin G, Brice A, Potter NT, Ross CA, Margolis RL: A repeat expansion in the gene encoding junctophilin-3 is associated with Huntington disease-like 2. Nat Genet 2001;29:377–378.
12 Holmes SE, O'Hearn E, Margolis RL: Why is SCA12 different from other SCAs? Cytogenet Genome Res 2003;100:189–197.
13 Lin X, Ashizawa T: SCA10 and ATTCT repeat expansion: clinical features and molecular aspects. Cytogenet Genome Res 2003;100:184–188.
14 Tapscott SJ: Deconstructing myotonic dystrophy. Science 2000;289:1701–1702.
15 Mankodi A, Logigian E, Callahan L, McClain C, White R, Henderson D, Krym M, Thornton CA: Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. Science 2000;289: 1769–1773.
16 Berul CI, Maguire CT, Aronovitz MJ, Greenwood J, Miller C, Gehrmann J, Housman D, Mendelsohn ME, Reddy S: DMPK dosage alterations result in atrioventricular conduction abnormalities in a mouse myotonic dystrophy model. J Clin Invest 1999;103:R1–R7.
17 Berul CI, Maguire CT, Gehrmann J, Reddy S: Progressive atrioventricular conduction block in a mouse myotonic dystrophy model. J Interv Card Electrophysiol 2000;4:351–358.
18 Klesert TR, Cho DH, Clark JI, Maylie J, Adelman J, Snider L, Yuen EC, Soriano P, Tapscott SJ: Mice deficient in Six5 develop cataracts: implications for myotonic dystrophy. Nat Genet 2000;25:105–109.
19 Sarkar PS, Appukuttan B, Han J, Ito Y, Ai C, Tsai W, Chai Y, Stout JT, Reddy S: Heterozygous loss of *Six5* in mice is sufficient to cause ocular cataracts. Nat Genet 2000;25:110–114.
20 Ranum L, Rasmussen P, Benzow K, Koob M, Day J: Genetic mapping of a second myotonic dystrophy locus. Nat Genet 1998;19:196–198.
21 Ricker K, Koch MC, Lehmann-Horn F, Pongratz D, Otto M, Heine R, Moxley III RT: Proximal myotonic myopathy: a new dominant disorder with myotonia, muscle weakness, and cataracts. Neurology 1994;44:1448–1452.

22 Thornton CA, Griggs RC, Moxley RT: Myotonic dystrophy with no trinucleotide repeat expansion. Ann Neurol 1994;35:269–272.
23 Ricker K, Koch M, Lehmann-Horn F, Pongratz D, Speich N, Reiners K, Schneider C, Moxley MR 3rd: Proximal myotonic myopathy. Clinical features of a multisystem disorder similar to myotonic dystrophy. Arch Neurol 1995;52:25–31.
24 Day JW, Roelofs R, Leroy B, Pech I, Benzow K, Ranum LPW: Clinical and genetic characteristics of a five-generation family with a novel form of myotonic dystrophy (DM2). Neuromuscul Disord 1999;9:19–27.
25 Ranum LPW, Day JW: Myotonic dystrophy: RNA pathogenesis comes into focus. Am J Hum Genet 2004;74:793–804.
26 Tapscott SJ, Thornton CA: Reconstructing myotonic dystrophy. Science 2001;293:816–817.
27 Liquori CL, Ikeda Y, Weatherspoon M, Ricker K, Schoser BG, Dalton JC, Day JW, Ranum LP: Myotonic dystrophy type 2: human founder haplotype and evolutionary conservation of the repeat tract. Am J Hum Genet 2003;73:849–862.
28 Day JW, Ricker K, Jacobsen JF, Rasmussen LJ, Dick KA, Kress W, Schneider C, Koch MC, Beilman GJ, Harrison AR, Dalton JC, Ranum LP: Myotonic dystrophy type 2: molecular, diagnostic and clinical spectrum. Neurology 2003;60:657–664.
29 Amack JD, Paguio AP, Mahadevan MS: Cis and trans effects of the myotonic dystrophy (DM) mutation in a cell culture model. Hum Mol Genet 1999;8:1975–1984.
30 Storbeck CJ, Drmanic S, Daniel K, Waring JD, Jirik FR, Parry DJ, Ahmed N, Sabourin LA, Ikeda JE, Korneluk RG: Inhibition of myogenesis in transgenic mice expressing the human DMPK 3′-UTR. Hum Mol Genet 2004;13:589–600.
31 Seznec H, Agbulut O, Sergeant N, Savouret C, Ghestem A, Tabti N, Willer JC, Ourth L, Duros C, Brisson E, Fouquet C, Butler-Browne G, Delacourte A, Junien C, Gourdon G: Mice transgenic for the human myotonic dystrophy region with expanded CTG repeats display muscular and brain abnormalities. Hum Mol Genet 2001;10:2717–2726.
32 Timchenko LT, Timchenko NA, Caskey CT, Roberts R: Novel proteins with binding specificity for DNA CTG repeats and RNA CUG repeats: implications for myotonic dystrophy. Hum Mol Genet 1996;5:115–121.
33 Miller JW, Urbinati CR, Teng-Umnuay P, Stenberg MG, Byrne BJ, Thornton CA, Swanson MS: Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. EMBO J 2000;19:4439–4448.
34 Fardaei M, Rogers MT, Thorpe HM, Larkin K, Hamshere MG, Harper PS, Brook JD: Three proteins, MBNL, MBLL and MBXL, co-localize in vivo with nuclear foci of expanded-repeat transcripts in DM1 and DM2 cells. Hum Mol Genet 2002;11:805–814.
35 Timchenko NA, Iakova P, Cai ZJ, Smith JR, Timchenko LT: Molecular basis for impaired muscle differentiation in myotonic dystrophy. Mol Cell Biol 2001;21:6927–6938.
36 Savkur RS, Philips AV, Cooper TA: Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. Nat Genet 2001;29:40–47.
37 Kanadia RN, Johnstone KA, Mankodi A, Lungu C, Thornton CA, Esson D, Timmers AM, Hauswirth WW, Swanson MS: A muscleblind knockout model for myotonic dystrophy. Science 2003;302:1978–1980.
38 Mankodi A, Takahashi MP, Jiang H, Beck CL, Bowers WJ, Moxley RT, Cannon SC, Thornton CA: Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. Mol Cell 2002;10:35–44.
39 Charlet BN, Savkur RS, Singh G, Philips AV, Grice EA, Cooper TA: Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. Mol Cell 2002;10:45–53.
40 Savkur RS, Philips AV, Cooper TA, Dalton JC, Moseley ML, Ranum LP, Day JW: Insulin receptor splicing alteration in myotonic dystrophy type 2. Am J Hum Genet 2004;74:1309–1313.
41 Ebralidze A, Wang Y, Petkova V, Ebralidse K, Junghans RP: RNA leaching of transcription factors disrupts transcription in myotonic dystrophy. Science 2004;303:383–387.
42 Ladd AN, Charlet N, Cooper TA: The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Mol Cell Biol 2001;21:1285–1296.

43 Roberts R, Timchenko NA, Miller JW, Reddy S, Caskey CT, Swanson MS, Timchenko LT: Altered phosphorylation and intracellular distribution of a $(CUG)_n$ triplet repeat RNA-binding protein in patients with myotonic dystrophy and in myotonin protein kinase knockout mice. Proc Natl Acad Sci USA 1997;94:13221–13226.
44 Timchenko NA, Patel R, Iakova P, Cai ZJ, Quan L, Timchenko LT: Overexpression of CUG triplet repeat-binding protein, CUGBP1, in mice inhibits myogenesis. J Biol Chem 2004;279:13129–13139.
45 Ho TH, Bundman D, Armstrong DL, Cooper TA: Transgenic mice expressing CUG-BP1 reproduce splicing mis-regulation observed in myotonic dystrophy. Hum Mol Genet 2005;14:1539–1547.
46 Timchenko LT, Miller JW, Timchenko NA, DeVore DR, Datar KV, Lin L, Roberts R, Caskey CT, Swanson MS: Identification of a $(CUG)_n$ triplet repeat RNA-binding protein and its expression in myotonic dystrophy. Nucleic Acids Res 1996;24:4407–4414.
47 Fardaei M, Larkin K, Brook JD, Hamshere MG: In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts. Nucleic Acids Res 2001;29:2766–2771.
48 Mankodi A, Urbinati CR, Yuan QP, Moxley RT, Sansone V, Krym M, Henderson D, Schalling M, Swanson MS, Thornton CA: Muscleblind localizes to nuclear foci of aberrant RNA in myotonic dystrophy types 1 and 2. Hum Mol Genet 2001;10:2165–2170.
49 Mankodi A, Teng-Umnuay P, Krym M, Henderson D, Swanson M, Thornton CA: Ribonuclear inclusions in skeletal muscle in myotonic dystrophy types 1 and 2. Ann Neurol 2003;54:760–768.
50 Mastaglia FL, Harker N, Phillips BA, Day TJ, Hankey GJ, Laing NG, Fabian V, Kakulas BA: Dominantly inherited proximal myotonic myopathy and leukoencephalopathy in a family with an incidental CLCN1 mutation. J Neurol Neurosurg Psychiatry 1998;64:543–547.
51 Buj-Bello A, Laugel V, Messaddeq N, Zahreddine H, Laporte J, Pellissier JF, Mandel JL: The lipid phosphatase myotubularin is essential for skeletal muscle maintenance but not for myogenesis in mice. Proc Natl Acad Sci USA 2002;99:15060–15065.
52 Faustino NA, Cooper TA: Pre-mRNA splicing and human disease. Genes Dev 2003;17:419–437.
53 Jiang H, Mankodi A, Swanson MS, Moxley RT, Thornton CA: Myotonic dystrophy type 1 is associated with nuclear foci of mutant RNA, sequestration of muscleblind proteins and deregulated alternative splicing in neurons. Hum Mol Genet 2004;13:3079–3088.
54 Philips AV, Timchenko LT, Cooper TA: Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. Science 1998;280:737–741.
55 Ladd AN, Stenberg MG, Swanson MS, Cooper TA: Dynamic balance between activation and repression regulates pre-mRNA alternative splicing during heart development. Dev Dyn 2005;233:783–793.
56 Harper PS: Myotonic Dystrophy (W.B. Saunders, London 2001).
57 Sergeant N, Sablonniere B, Schraen-Maschke S, Ghestem A, Maurage CA, Wattez A, Vermersch P, Delacourte A: Dysregulation of human brain microtubule-associated tau mRNA maturation in myotonic dystrophy type 1. Hum Mol Genet 2001;10:2143–2155.
58 Faustino NA, Cooper TA: Identification of putative new splicing targets for ETR-3 using sequences identified by systematic evolution of ligands by exponential enrichment. Mol Cell Biol 2005;25:879–887.
59 Filippova GN, Thienes CP, Penn BH, Cho DH, Hu YJ, Moore JM, Klesert TR, Lobanenkov VV, Tapscott SJ: CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DM1 locus. Nat Genet 2001;28:335–343.
60 Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R, Hills J, Grigsby J, Gage B, Hagerman PJ: Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 2001;57:127–130.
61 Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen LA, Nieuwenhuizen IM, Schrier M, van Unen L, Tassone F, Hoogeveen AT, Hagerman PJ, Mientjes EJ, Oostra BA: The *FMR1* CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet 2003;12:949–959.
62 Van Dam D, Errijgers V, Kooy RF, Willemsen R, Mientjes E, Oostra BA, De Deyn PP: Cognitive decline, neuromotor and behavioural disturbances in a mouse model for fragile-X-associated tremor/ataxia syndrome (FXTAS). Behav Brain Res 2005;162:233–239.
63 Jin P, Zarnescu DC, Zhang F, Pearson CE, Lucchesi JC, Moses K, Warren ST: RNA-mediated neurodegeneration caused by the fragile X premutation rCGG repeats in *Drosophila*. Neuron 2003;39:739–747.

64 Koob MD, Moseley ML, Schut LJ, Benzow KA, Bird TD, Day JW, Ranum LPW: An untranslated CTG expansion causes a novel form of spinocerebellar ataxia (SCA8). Nat Genet 1999;21:379–384.
65 Day JW, Schut LJ, Moseley ML, Durand AC, Ranum LPW: Spinocerebellar ataxia type 8: clinical features in a large family. Neurology 2000;55:649–657.
66 Koob MD, Benzow KA, Bird TD, Day JW, Moseley ML, Ranum LPW: Rapid cloning of expanded trinucleotide repeat sequences from genomic DNA. Nat Genet 1998;18:72–75.
67 Nemes JP, Benzow KA, Moseley ML, Ranum LPW, Koob MD: The SCA8 transcript is an antisense RNA to a brain-specific transcript encoding a novel actin-binding protein (KLHL1). Hum Mol Genet 2000;9:1543–1551. [Correction/Addition Hum Mol Genet 1549:2777].
68 Benzow KA, Koob MD: The *KLHL1*-antisense transcript (*KLHL1AS*) is evolutionarily conserved. Mamm Genome 2002;13:134–141.
69 Ikeda Y, Shizuka M, Watanabe M, Okamoto K, Shoji M: Molecular and clinical analyses of spinocerebellar ataxia type 8 in Japan. Neurology 2000;54:950–955.
70 Cellini E, Nacmias B, Forleo P, Piacentini S, Guarnieri BM, Serio A, Calabro A, Renzi D, Sorbi S: Genetic and clinical analysis of spinocerebellar ataxia type 8 repeat expansion in Italy. Arch Neurol 2001;58:1856–1859.
71 Topisirovic I, Dragasevic N, Savic D, Ristic A, Keckarevic M, Keckarevic D, Culjkovic B, Petrovic I, Romac S, Kostic VS: Genetic and clinical analysis of spinocerebellar ataxia type 8 repeat expansion in Yugoslavia. Clin Genet 2002;62:321–324.
72 Moseley ML, Schut LJ, Bird TD, Koob MD, Day JW, Ranum LPW: SCA8 CTG repeat: en masse contractions in sperm and intergenerational sequence changes may play a role in reduced penetrance. Hum Mol Genet 2000;9:2125–2130.
73 Stevanin G, Herman A, Durr A, Jodice C, Frontali M, Agid Y, Brice A: Are $(CTG)_n$ expansions at the SCA8 locus rare polymorphisms? Nat Genet 2000;24:213; discussion 215.
74 Moseley ML, Weatherspoon M, Rasmussen L, Day JW, Ranum LPW: SCA8 BAC transgenic mice have a progressive and lethal neurological phenotype demonstrating pathogenicity of the CTG expansion. Am J Hum Genet 2002;(suppl 7):A64.
75 Mutsuddi M, Marshall C, Benzow K, Koob M, Rebay I: The spinocerebellar ataxia 8 noncoding RNA causes neurodegeneration and associates with staufen in *Drosophila*. Curr Biol 2004;14: 302–308.
76 Mosemiller AK, Dalton JC, Day JW, Ranum LPW: Molecular genetics of spinocerebellar ataxia type 8 (SCA8). Cytogenet Genome Res 2003;100:175–183.
77 Janzen MA, Moseley ML, Benzow KA, Day JW, Koob MD, Ranum LPW: Limited expression of SCA8 is consistent with cerebellar pathogenesis and toxic gain of function RNA model. Am J Hum Genet 1999;65:A267.
78 Jansen G, Groenen PJTA, Bachner D, Jap PHK, Coerwinkel M, Oerlemans F, van den Broek W, Gohlsch B, Pette D, Plomp JJ, Molenaar PC, Nederhof MGJ, van Echted CJA, Dekker M, Berns A, Hameister H, Wieringa B: Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. Nat Genet 1996;13:316–324.
79 Rasmussen A, Matsuura T, Ruano L, Yescas P, Ochoa A, Ashizawa T, Alonso E: Clinical and genetic analysis of four Mexican families with spinocerebellar ataxia type 10. Ann Neurol 2001;50:234–239.
80 Lin X, Ashizawa T: Recent progress in spinocerebellar ataxia type-10 (SCA10). Cerebellum 2005;4:37–42.
81 Matsuura T, Yamagata T, Burgess DL, Rasmussen A, Grewal RP, Watase K, Khajavi M, McCall AE, Davis CF, Zu L, Achari M, Pulst SM, Alonso E, Noebels JL, Nelson DL, Zoghbi HY, Ashizawa T: Large expansion of the ATTCT pentanucleotide repeat in spinocerebellar ataxia type 10. Nat Genet 2000;26:191–194.
82 Marz P, Probst A, Lang S, Schwager M, Rose-John S, Otten U, Ozbek S: Ataxin-10, the spinocerebellar ataxia type 10 neurodegenerative disorder protein, is essential for survival of cerebellar neurons. J Biol Chem 2004;279:35542–35550.
83 O'Hearn E, Holmes SE, Calvert PC, Ross CA, Margolis RL: SCA-12: tremor with cerebellar and cortical atrophy is associated with a CAG repeat expansion. Neurology 2001;56:299–303.
84 Holmes SE, O'Hearn EE, McInnis MG, Gorelick-Feldman DA, Kleiderlein JJ, Callahan C, Kwak NG, Ingersoll-Ashworth RG, Sherr M, Sumner AJ, Sharp AH, Ananth U, Seltzer WK, Boss MA,

Vieria-Saecker AM, Epplen JT, Riess O, Ross CA, Margolis RL: Expansion of a novel CAG trinucleotide repeat in the 5′ region of *PPP2R2B* is associated with SCA12. Nat Genet 1999;23:391–392.
85 Holmes SE, Hearn EO, Ross CA, Margolis RL: SCA12: an unusual mutation leads to an unusual spinocerebellar ataxia. Brain Res Bull 2001;56:397–403.
86 Ranum LPW, Day JW: Pathogenic RNA repeats: an expanding role in genetic disease. Trends Genet 2004;20:506–512.

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Telomeres and Telomerase in Stem Cells during Aging and Disease

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Abstract

Cell cycle checkpoints induced by telomere dysfunction represent one of the major in vivo tumor suppressor mechanisms preventing cancer but at the same time provoking age dependent decline in self-renewal and regeneration of tissues and organs. On the other hand, telomere shortening contributes to the initiation of cancer by inducing chromosomal instability. Telomere function and telomerase activity are mainly associated with actively proliferating cells. Since stem cells are continuously proliferating throughout lifetime, it is of great interest to explore the role of telomeres and telomerase in stem cells. Although most stem cell compartments express telomerase, the level of telomerase activity is not sufficient to maintain telomere length of stem cells during aging. Stem cells appear to have tighter DNA-damage checkpoint control in comparison to somatic cells, which may reflect the need to protect this long lasting cell compartment against malignant transformation. These enhanced checkpoint responses may have a detrimental impact on stem cell function, by causing increased sensitivity towards senescence or apoptosis induced by telomere shortening. This review summarizes our knowledge on telomere dynamics and its functional impact on stem cells during aging and transformation.

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Telomeres, Telomerase and Senescence: How Are They Interconnected?

Telomeres are complex nucleo-protein structures composed of linear arrays of double-stranded TTAGGG repeats, a 3′ single strand overhang, and associated telomere binding proteins [1]. The main function of telomeres is to cap chromosomal ends, which allows cells to distinguish natural chromosomal ends from DNA breaks within a chromosome thereby protecting chromosomes from end to end fusions, degradation, and instability [2, 3]. Telomerase can

recognize the extreme terminus of a G-rich strand of an existing telomere and elongate it in the 5′-to-3′ direction [2, 4, 5]. For the synthesis of telomeres two components of the telomerase enzyme are essential: (1) the RNA component (TERC) that serves as a template for the addition of telomeric repeats [6, 7], and (2) telomerase reverse transcriptase (TERT), which is the catalytic subunit of the enzyme [8–10]. In addition, a number of proteins associated with the telomerase enzyme complex are necessary for proper telomerase function [5, 11].

Due to processing of DNA-termini during S-phase of the cell cycle, the 3′ telomeric DNA end remains slightly longer than the 5′ end, leaving a protruding single-strand. Aided by telomere binding proteins, the double-stranded telomere loops back to tuck its single-stranded 3′ terminus into the proximal duplex telomeric DNA to form a loop structure (T-loop) [12]. In addition, telomeres can form g-quadruplex structures [13, 14]. These tertiary structures of telomeres appear to be essential for telomere capping function [2]. A variety of telomere single- and double-strand binding proteins have been shown to be important for maintaining the T-loop structure. In addition, these proteins play a role in regulating telomerase and proteins involved in DNA-damage recognition, signaling, and repair [5, 15].

Telomerase is active – and plays an essential role in telomere elongation – during embryogenesis [16, 17]. Postnatal TERT expression is suppressed in most somatic tissues [16] leading to telomere shortening during each cell division due to both the 'end replication problem' of DNA-polymerase [18] and postreplication processing of telomere ends during S-phase [19]. In human cells telomeres shorten at a rate of 50–100 base pairs per cell division [20]. When telomeres reach a critically short length, cells enter a non-proliferating state of permanent cell cycle arrest termed 'senescence' [21, 22]. In line with the telomere hypothesis of senescence, ectopic expression of human TERT (hTERT) extends the proliferative life-span of many human cell types including fibroblasts [23], osteoblasts [24], hepatocytes [25] and neural progenitor cells [26].

The telomere hypothesis of replicative senescence indicates that progressive telomere shortening eventually triggers an alteration in telomere structure, which leads to the deprotection of chromosomal ends. These exposed ends are then recognized as DNA-breaks leading to activation of double-strand and single-strand DNA damage responses involving the activation of ATM/ATR/p53/CHK2/p21-pathways consequently producing cellular senescence [27–31].

In adult humans, telomerase activity is restricted to cell types that are undifferentiated and highly proliferative, such as activated lymphocytes [32, 33], germ cells [16], and certain progenitor cells [34]. In tissues without detectable telomerase activity, the telomerase RNA transcript is usually present, but the TERT mRNA is suppressed [35–37]. Some reports suggested that TERT is transiently expressed in some somatic human cells during cell differentiation

and during S-phase of cell division although the physiological relevance of this phenomenon remains to be established in vivo [38, 39].

Mouse Models of Telomere Dysfunction

In contrast to humans, somatic cells in laboratory mouse strains show constitutive telomerase activity and very long telomeres. However, genetic experiments in which telomerase components were disrupted in mouse models have revealed an essential role of telomere length maintenance for the overall fitness, reserve, and well being of the aging organism [40, 41]. Deletion of TERC in mice led to loss of telomerase activity and progressive telomere shortening in all tissues. Due to very long telomeres in mice, first generation of mTERC$^{-/-}$ mice do not show any gross pathological phenotype, indicating that telomerase function is not required for development and organ homeostasis in the presence of long telomere reserves [3]. Telomere shortening in successive generations of mTERC$^{-/-}$ mice was associated with premature aging predominantly affecting organ systems with high rates of cell turnover, e.g. the skin, the intestinal epithelium, and the hematopoietic system [40–42]. In addition, mTERC$^{-/-}$ mice showed an increased rate of tumor initiation and a reduced lifespan [40, 43, 44]. The late generation mice had significant reduction in telomere length compared to early generation animals, thus the marked phenotypes in these animals likely reflect a critical role of telomere length, rather than telomerase activity per se in regulating cell proliferation and organ homeostasis. Telomere shortening is heterogeneous at the cellular level and the impaired regenerative capacity in mTERC$^{-/-}$ mice was due to a reduction of the proliferating pool of organ cells whose critically short telomeres restricted cell cycle entry [45]. In addition to telomerase knockout mouse models, telomerase transgenic mice have been produced. K5-TERT mice, which overexpress TERT in the skin, showed faster wound healing but increased tumorigenesis compared to wild-type controls [46]. Surviving cancer-free TERT-transgenic mice had an extended maximum lifespan compared to wild-type littermates [47] supporting the hypothesis that extinction of telomerase activity may have dual effects on the organismal level by promoting tumor suppression but impairing the regenerative reserve during aging (see below).

Telomerase and Telomere Shortening in Human Stem Cells

In contrast to most somatic tissues, telomerase activity is present in adult humans in stem cells and progenitor cells including dermal stem cells at the

basal layer of the epidermis [48], hematopoietic stem cells (HSCs) in the bone marrow (BM) [34, 49, 50], intestinal stem cells in the basal crypts [37, 51], neural stem cells [52], and cardiac stem cells [53]. One exception are mesenchymal stem cells (MSCs), which appear to be telomerase negative [54, 55]. Some reports suggested that the level of telomerase activity is low in true stem cells but high in progenitor compartments undergoing massive rounds of cell divisions [34, 56]. However, telomerase activity in true stem cells appears to correlate with self-renewal capacity [56].

Despite the presence of readily detectable levels of telomerase in human HSCs, a number of studies have demonstrated that telomeres do shorten in peripheral blood cells during aging and after BM transplantation [for review see 57, 58]. Direct evidence for telomere shortening in HSCs has come from a study showing telomere shortening in purified HSCs from adult BM compared to purified stem cells from fetal liver or newborn umbilical cord blood [59]. Transplantation of limited numbers of HSCs during BM transplantation exerts a high proliferative demand on stem cells. After BM transplantation, accelerated telomere shortening was observed in the first year [60, 61]. However, there is no direct evidence linking BM failure after HSC transplantation to the telomere length of donor stem cells.

Functional Impact of Telomere Shortening on Stem Cells

Experimental data in mice have revealed that HSCs have limited ability to repopulate irradiated recipients in serial transplantation experiments [62]. There is experimental evidence indicating that stem cell function is reduced during aging [63, 64]. However, the molecular mechanisms underlying stem cell aging have yet to be explored. As noted above, telomere shortening has been demonstrated in human hematopoietic system during aging, pointing to one potential mechanism of stem cell aging. In normal mice HSCs can successfully engraft lethally irradiated recipients for at least 4 rounds of serial transplantations. In contrast, telomerase-deficient HSCs showed a reduced regenerative capacity and could only be serially transplanted for 2 rounds [65]. In these experiments, the rate of telomere shortening in donor cells from mTERC$^{-/-}$ mice was 2-fold increased compared to wild-type HSCs. In line with these experiments, mTERC$^{-/-}$ HSC showed a reduced long-term repopulating capacity in competitive transplantation experiments [66]. Together, these data indicate that telomerase ensures stem cell replicative capacity by reducing the rate of telomere shortening during cell division. However, overexpression of telomerase in wildtype mice did not extend the HSC transplantation capacity, although telomere length was more stable during serial transplantations compared

to non-transgenic control HSC [67]. A possible explanation is that telomere-independent barriers may limit the transplantation capacity of HSCs, in addition to the telomere-dependent mechanisms, which were observed in mTERC$^{-/-}$ HSCs.

The above studies have given experimental evidence that telomere shortening can limit the replicative capacity of HSCs. In agreement with these data, aging mTERC$^{-/-}$ mice developed anemia and leucopenia [41] and BM cells from mTERC$^{-/-}$ mice showed a reduced colony forming capacity [42, 66]. Overall, it appears that telomere dysfunction led to the depletion of stem cells such as germ cells and HSCs in these animals [42, 68]. It has yet to be determined to what extent telomere shortening can impact on stem cell function in other organ systems, although some of the organ-phenotypes in aging mTERC$^{-/-}$ mice (see above) might be in part due to impaired stem cell function e.g. villus atrophy of the intestine, impaired proliferation of the cells of the immune system, alopecia, decreased wound healing, among others [40, 42]. The first experimental evidence for a direct effect of telomere shortening on organ stem cells came from studies in mTERC$^{-/-}$, ATM$^{-/-}$ compound mutant mice [28]. ATM$^{-/-}$ mice did not show the classical ataxia phenotype observed in humans with ATM-gene mutation. However, neural stem cells from mTERC$^{-/-}$, ATM$^{-/-}$ double knockout mice showed reduced proliferation compared to those from mTERC$^{+/+}$, ATM$^{-/-}$ mice both in vivo and in vitro [28]. In addition, telomere shortening and telomere dysfunction inhibited proliferation and mobilization of epidermal stem cells from the dermal stem cell niche [69]. Together, these data indicate that telomere shortening can affect the replicative capacity of organ specific stem cells during aging.

It is not yet clear how telomere shortening affects stem cell fitness. It may impinge on self-renewal, differentiation or progenitor cell proliferation [56]. Studies in telomerase knockout mice indicate that telomere shortening might reduce the absolute number of stem cells and the proliferative capacity of stem cells (see above). Since impaired function of stem cells was not observed in first generation mTERC$^{-/-}$ with long telomere reserves, the stem cell defects in late generation mTERC$^{-/-}$ mice are likely induced by telomere shortening and not by the absence of telomerase per se. However, in vitro experiments on murine MSCs have shown that telomerase enzyme itself was necessary for differentiation of MSCs into adipocytes and chondrocytes [70]. In addition, it has been reported that overexpression of TERT enhanced self-renewal, resistance to oxidative stress, and the differentiation potential of murine embryonic stem cells [71]. Moreover, TERT overexpression in the epidermis of transgenic mice resulted in increased stem cell proliferation and hair growth compared to wildtype mice [69, 72]. Therefore, telomere-independent effects of telomerase on stem cell function appear to be relevant.

One working hypothesis for the study of stem cell aging is that telomere shortening impairs functionality and reduces the total number of stem cells (see above). However, a direct connection between stem cell aging, telomere shortening, and induction of DNA damage signals has yet to be established. It is now possible to purify pluripotent stem cells of various organ systems. These methods should facilitate the analysis of DNA-breaks, telomere length and senescence markers in stem cell populations during aging. Several lines of evidence indicate that stem cells are more sensitive to DNA-damage compared to somatic cells [73–75]. For example, cytotoxicity to stem cells limits the maximum tolerable doses of irradiation (IR) and chemotherapy of cancer patients. The DNA damage response in stem cells appears to be fundamentally different from responses observed in somatic cells [73–75]. Experimental data have revealed that embryonic stem cells (ESCs) are hypersensitive to IR and other DNA-damaging agents, responding with induction of p53-independent apoptosis [73]. It has been shown that the G_1 checkpoint – one of the major checkpoints in response to DNA damage in somatic cells – is virtually absent in ESCs [74]. p53 protein appears not to be fully functional in ESCs due to its predominantly cytoplasmic localization and inefficient translocation to the nucleus in response to DNA damage [74]. Similarly, the function of Chk2 appears to be hampered in ESCs compared to somatic cells [73]. The induction of apoptosis instead of cell cycle arrest in stem cells might be beneficial to ensure removal of stem cells with damaged DNA, thereby maintaining a pristine cell population [73]. In addition to the induction of apoptosis, DNA damage can also activate a differentiation checkpoint in stem cells [75], which might be a backup mechanism to limit the lifespan of genetically damaged stem cells. It remains to be tested whether the above findings on DNA damage checkpoints in embryonic stem cells apply to adult stem cells as well.

Given their sensitivity to DNA damaging agents, it is plausible to suggest that stem cells may also show enhanced sensitivity to telomere shortening compared to somatic cells. One study has shown that there are intrinsic differences between embryonic and adult neural stem cells in their response to telomere shortening. Adult neural stem cells (NSCs) with shorter telomeres show impaired cell proliferation. In contrast, embryonic NSCs with similarly short telomeres developed chromosomal instability but did not show reduced proliferation despite an accumulation of nuclear p53 levels [76]. In line with the hypothesis that stem cells are highly sensitive to telomere shortening, humans suffering from the rare disease Dyskeratosis congenita (DKC) die from BM failure. The pathology of DKC has been related to compromised telomerase function leading to a defect in telomere maintenance [77, 78]. DKC is characterized by defects in highly regenerative tissues such as skin and

BM, and an increased risk of malignant tumors [79]. An autosomal-dominant form of DKC has been linked to mutation in the gene encoding human TERC (hTERC) [80]. Patients with this form of the disease are more severely affected in later generations possibly due to the inheritance of shortened telomeres [81]. Lymphocytes from patients with autosomal dominant DKC overexpress markers of senescence including CD57 and Fas receptor indicating that short telomeres trigger a senescence response in this cellular compartment [82]. Further examples of BM failure in humans induced by telomere shortening are aplastic anemia and Fanconi's anemia (FA) [83–85]. Mutations in hTERT leading to haploinsufficiency have recently been identified in a subset of patients with this condition [86]. In addition, granulocytes from these patients showed progressive telomere shortening due to extensive proliferation of HSCs [87, 88].

Cancer, Telomeres, and Telomerase

Several studies in diverse model systems have shown that telomeres and telomerase play critical roles in the maintenance of chromosomal integrity [3, 44, 89–92]. The original telomere-hypothesis indicated that suppression of telomerase activity and limitation of cell proliferation by senescence represent a tumor suppressor mechanism [93, 94]. In line with the classical view, telomere shortening suppressed tumor progression in mTERC$^{-/-}$ mice by induction of apoptosis and impairment of tumor cell proliferation [22, 44, 93, 94]. However, studies in mTERC$^{-/-}$ mice revealed a dual role of shortened telomeres during tumorigenesis. In successive generations of TERC$^{-/-}$ mice crossed with APC^{Min} mice – a model of intestinal tumor formation – progressive telomere dysfunction led to an increase in tumor initiation, yet to a significant decline in the multiplicity and size of macroscopic adenomas [44]. Similarly telomere shortening in mTERC$^{-/-}$ mice increased tumor initiation but suppressed tumor progression during hepatocarcinogenesis [95]. Together, these data from mTERC$^{-/-}$ mice have suggested a new model of the role of telomeres in carcinogenesis, which implicates that telomere dysfunction enhances early carcinogenesis by inducing chromosomal instability, while telomerase activation is necessary to restore genomic stability to a level permitting tumor progression [96, 97]. In line with the role of p53 in the senescence pathway, p53 deletion rescued organ phenotypes of telomere dysfunction in late generation mTERC$^{-/-}$ mice [27]. Interestingly, p53 heterozygous mice with telomere dysfunction demonstrated a significant shift in the tumor spectrum showing an increased incidence of epithelial cancers, which exhibited complex cytogenetic alterations [98]. The shift in tumor spectrum to cytogenetically unstable epithelial

cancers in this model indicated that telomere shortening and loss of p53 function might cooperate to increase age-related cancer formation. It is notable that some human cancers, such as pancreatic adenocarcinoma, show telomere shortening as the first detectable molecular alteration at the earliest stages of neoplasia and subsequently lose p53 checkpoint function during multistage tumor progression.

In addition to cell autonomous mechanisms leading to induction of DNA damage response and chromosomal instability, organismal telomere shortening could have systemic effects on tumorigenesis. For example, it has been shown that telomere shortening impaired angiogenesis and thereby inhibited tumor progression [99]. Another potential effect of telomere shortening on tumorigenesis is diminished immune surveillance of nascent cancers associated with telomere length-related senescence of immunocytes [100, 101].

In vitro and in vivo studies have revealed tumor promoting effects of TERT [102, 103]. It has been shown that TERT expression was necessary for transformation of primary human fibroblasts in vitro [103]. However, primary cells with long telomere reserves were capable of transformation without ectopic expression of TERT or TERC indicating that the main function of TERT during transformation is telomere stabilization [3, 104, 105]. Some studies, however, indicated that TERT might have additional, telomere length independent effects on tumorigenesis. It has been shown that ectopic expression of a mutant version of hTERT, which is insufficient for telomere stabilization yet retains catalytic activity, can impart the tumorigenic phenotype of telomerase-negative human cancer cell lines [106]. In addition, a hairpin siRNA specifically targeting human telomerase RNA rapidly inhibited growth of human cancer cells independent of p53 status and independent of telomere length [107]. One possible explanation is that telomerase activity affects the length of the telomere 3′ overhang, which plays an essential role in maintaining proper telomere structure, thereby modulating growth arrest or apoptosis independent of absolute telomere length [38, 108]. Another possibility is that telomerase may affect proliferation of cells not only by stabilizing telomeres, but also by affecting the expression of growth-promoting genes. In agreement with this hypothesis, telomerase RNA knock-down experiments in cancer cells induced changes in global gene expression, which included suppression of specific genes implicated in angiogenesis and metastasis [109]. Similarly, it has been shown that TERT can act as a transcription factor regulating the expression of many genes involved in cell cycle control [110]. In transgenic mouse models, overexpression of TERT induced carcinogenesis in different cellular compartments [102, 111]. Since mice have very long telomeres, this effect appears telomere length independent. However, tumor initiation by TERT overexpression was abolished in $TERC^{-/-}$ TERT-transgenic mice indicating that tumor initiation requires

TERT-TERC complex formation – and, potentially, proficient telomerase activity [111]. Together, the main function of telomerase during transformation is telomere stabilization but telomerase enzyme appears to have additional telomere length independent effects on transformation.

As discussed above, telomerase activity appears to be regulated in a biphasic manner during tumor progression, with low or absent activity detected in the premalignant lesions and pronounced telomerase activity in most malignant tumors. The mechanistic role of telomerase reactivation in multistage tumorigenesis has yet to be investigated in vivo. A prevailing hypothesis is that telomere shortening and loss of p53 may facilitate the initiating stages of malignant transformation by enabling the survival of genetically unstable cells, whereas subsequent telomerase reactivation might be necessary for creating a degree of genomic stabilizing and thereby permitting tumor cell proliferation and tumor progression [96]. The development of refined mouse models may provide key insights into the evolving roles of telomeres and telomerase in cancer pathogenesis.

Telomeres and Telomerase in Cancer Stem Cells

Stem cells undergo a life long exposure to damage from several intracellular and extracellular sources, such as reactive oxygen species (ROS) and genetic damage, replication errors, etc. Therefore, although rare in number, stem cells are susceptible to accumulating molecular alterations leading to malignant transformation. Tighter DNA damage checkpoints, and the likely stricter response to telomere dysfunction (see above) of stem cells compared to somatic cells may reflect the need for improved elimination of mutant cells. While tighter checkpoint control might help to prevent stem cell cancer in young age, it may contribute to regenerative exhaustion during aging (see above). Deducing from the cancer phenotypes in mTERC$^{-/-}$ mice, it appears that telomere shortening and telomerase reactivation could have a dual role in the formation of cancers derived from stem cells such as hematopoietic malignancies.

A variety of studies on hematopoietic malignancies have shown a correlation between shortened telomeres and high telomerase activity with disease progression and severity (table 1) [for review see 112]. With regard to telomerase activity, slightly increased levels of activity (2–5-fold) were found in chronic phase (CP) of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), polycythemia vera (PV), and myelodysplastic syndromes (MDS). However, highly elevated levels (50–100-fold) of telomerase activity were present during disease progression and were linked to acute disease stages in AML and CML [113]. Similarly, CLL patients show low telomerase activity in early disease stages but increased

Table 1. Telomere length and telomerase activity in bone marrow failure diseases and hematopoietic neoplasia

Disease	Telomerase	Telomere	Prognostic factors	Reference no.
Aplastic anemia	Haploinsufficiency due to mutations of TERC or TERT in some cases	Shortened	Severe telomere shortening correlates with chromosomal instability	83, 86–88
Fanconi anemia	Increased 5 fold	Accelerated rate of shortening		84–86
Dyskeratosis congenita	Mutation of TERC in autosomal dominant form	Progressive shortening	Disease anticipation correlates with short telomeres	80–82
Acute leukemia	High activity (>10 fold) in >75%	Shortened, altered expression of TRF1, TRF2 and Tin2	Level of telomerase is negative prognostic factor, decrease of telomerase activity and increase in telomere length correlate to remission state, telomere shortening correlates to chromosomal instability	113–117
CML, chronic phase	Normal or slightly increased (2–5 fold)	Shortened	Telomere shortening and telomerase activity correlate with disease progression (accelerated phase, blast phase); Telomere shortening correlates with prognostic score at diagnosis and proceeds rapidly during progression of chronic myeloid leukemia	116, 123–126
CML, acute phase	High activity in >40%, 10–50 fold	Extremely short		
B-CLL, early stage	Normal	Shortened	High rate of telomere shortening is a negative prognostic factor (high risk); High level of telomerase activity is negative prognostic factor	127–130
B-CLL, late stage	2–4 fold higher levels	Shorter than at early stage		

Table 1. (continued)

Disease	Telomerase	Telomere	Prognostic factors	Reference no.
MDS	Normal, slightly increased	Shortened	Telomere shortening is negative prognostic factor, related to complex cytogenetic abnormalities, anemia, and leukemic transformation	117, 120–122
NHL	High activity in 80% of samples	Significantly shorter	Level of telomerase activity correlates to grading and progression	131–134
Hodgkin's disease	Lack of telomerase activity in most cases, possibly caused by presence of eosinophils			112, 132, 135

levels in late disease stages [114] and telomerase activity was identified as a prognostic marker indicating CLL progression [115] and overall survival [116, 117]. Telomerase activity was also high in most of the patients with non-Hodgkin's lymphoma (NHL) [118], and the level of telomerase activity correlated to grading and disease progression [119, 120]. The only hematologic neoplasia that shows no tight correlation between telomerase and disease progression is Hodgkin's disease (HD) [118]. However, there is evidence that the level of telomerase might be underestimated in HD due to infiltration of eosinophils, which seem to express inhibitors interfering with the in vitro measurement of telomerase activity [121].

In addition to the correlation between level of telomerase activity and disease progression, there is also evidence for a correlation between telomerase activity and cytogenetic instability in hematopoietic malignancies, e.g. in CML [122] and MDS [123] patients. In MDS, some patients had cytogenetic changes without elevation of telomerase activity, but post-MDS AML patients showed elevated telomerase activity in correlation with increased rates of cytogenetic changes [123]. It remains to be determined whether telomerase activity is simply necessary for progressive proliferation of genetically instable cells or whether it plays an active role in evolution of cytogenetic instability. A variety of publications have described a correlation between the level of telomerase activity and poor prognosis of hematologic malignancies [112, 115, 124] indicating that telomerase is a negative prognostic marker and a potential therapeutic target.

Apart from the data on telomerase activation during progression of HSC-malignancies, there is accumulating evidence that telomere shortening represents a risk factor for stem cell transformation (table 1). MDS is characterized by cytopenia in the peripheral blood and dysplastic features in the hematopoietic cells. Since approximately 30% of patients show progression into AML, MDS is seen as a preleukemic state [125]. Telomere shortening is associated with genome instability in MDS and post-MDS AML [126]. MDS patients with shortened telomeres had significantly higher percentage of marrow blasts, a higher incidence of cytogenetic abnormalities, an increased risk of leukemic transformation, and a poorer prognosis compared to patients with longer telomeres [127, 128]. Another example is CML, which is a clonal disease with specific cytogenetic changes including the Philadelphia (Ph) translocation [129]. Telomere shortening was associated with disease history and disease progression in CML [122, 130, 131], and patients in late chronic phase had significantly shorter telomeres than those assessed in earlier chronic phase [132]. In agreement with this hypothesis significantly shorter telomeres were seen in the acute blast phase of end stage CML indicating that malignant clones arose from genetically unstable cells with critically short telomeres [122]. Similarly, telomere shortening of lymphatic cells was present in the vast majority of NHL [133] and lymphocyte telomere shortening has been identified as a negative prognostic factor of overall survival in B cell CLL (B-CLL) [116, 117].

In addition to telomere shortening and telomerase activation, a disruption of the normal telomere structure might influence disease progression in hematopoietic neoplasia. Consistent with this idea there is an increasing number of reports showing an altered expression of telomere binding proteins during the time course of hematopoietic carcinogenesis [134, 135].

Outlook

Experimental studies over the past years have revealed clear evidence that telomeres and telomerase have an impact on the function and transformation of stem cells. It appears that stem cells have tighter checkpoint responses to DNA damage, which could make these cells more sensitive to senescence and apoptosis induced by telomere dysfunction (fig. 1). Tighter checkpoint controls in stem cells might have evolved to protect this long lasting cell population against accumulation of genetic damage and transformation. The detrimental consequence of this protection may be the exhaustion of regenerative potential in response to aging and chronic disease. Since loss of checkpoint function is a basic mechanism in tumor development, it will be crucial for cancer biologists to characterize the

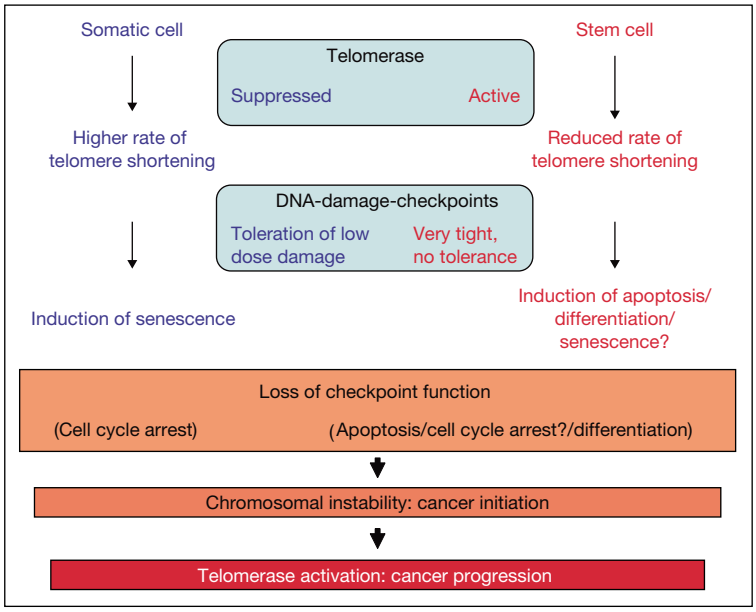

Fig. 1. Model on differences in telomere biology of stem cells and somatic cells. In contrast to somatic cells, stem cells express telomerase, which slows the rate of telomere shortening compared to somatic cells. This mechanism might be necessary to assure the high proliferative capacity of stem cells but does not completely prevent telomere shortening in stem cells during aging [58]. Stem cells have tighter DNA-damage checkpoints compared to somatic cells [73–75]. Somatic cells show 3–10 telomere free chromosomal ends when they enter senescence [21, 30]. Since stem cells have tighter DNA damage checkpoint responses it appears likely that a lower number of telomere free ends will lead to checkpoint activation in stem cells. DNA-damage induces different outcomes in stem cells compared to somatic cells: Induction of apoptosis or differentiation in stem cells vs. cell cycle arrest in somatic cells. Telomere induced senescence is similar to a cell cycle arrest induced by DNA damage in somatic cells. Whether stem cells undergo replicative senescence has not yet been clearly demonstrated. Loss of DNA-/telomere-damage induced checkpoint responses elicits progressive chromosomal instability in both cell types (somatic cells and stem cells), eventually leading to tumor initiation. In contrast, telomerase activation appears to be required for tumor progression in both compartments. However, the molecular mechanisms leading to telomerase reactivation might be different in both cell types since non-transformed stem cells express detectable levels of telomerase activity whereas most somatic cells do not express telomerase.

molecular pathways governing DNA-damage checkpoints induced by telomere dysfunction in stem cells. The data on hematopoietic malignancies indicate that telomere shortening and telomerase activation have dual effects on stem cell transformation. On the one hand telomere shortening leads to chromosomal instability and increases the risk of malignant transformation, on the other hand

telomerase activation and telomere stabilization appear to be necessary for proliferation and survival of genetically instable, malignant stem cells. It remains to be elucidated whether these observations from hematologic neoplasia can be translated to stem cells in solid organ tumors. The identification of malignant stem cells in cancers other than the hematopoietic system, such as breast and brain cancer, provide increasing evidence implying that stem cells might be the cell type of origin of different types of cancer. However, not much is known about telomeres and telomerase in cancer stem cells compared to the bulk of non-stem-cell cancer cells. Understanding telomere dynamics and telomerase regulation in this critical cell compartment could help to identify new therapeutic targets for the treatment and prevention of cancer.

References

1 Blackburn EH: Structure and function of telomeres. Nature 1991;350:569–573.
2 Blackburn EH: Switching and signaling at the telomere. Cell 2001;106:661–673.
3 Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW: Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997;91:25–34.
4 Greider CW, Blackburn EH: Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell 1985;43:405–413.
5 Smogorzewska A, de Lange T: Regulation of telomerase by telomeric proteins. Annu Rev Biochem 2004;73:177–208.
6 Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J: The RNA component of human telomerase. Science 1995;269:1236–1241.
7 Blasco MA, Rizen M, Greider CW, Hanahan D: Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. Nat Genet 1996;12:200–204.
8 Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR: Telomerase catalytic subunit homologs from fission yeast and human. Science 1997;277:955–959.
9 Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, Weinberg RA: hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 1997;90:785–795.
10 Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR: Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 1997;276:561–567.
11 Kim JH, Park SM, Kang MR, Oh SY, Lee TH, Muller MT, Chung IK: Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. Genes Dev 2005;19:776–781.
12 de Lange T: Protection of mammalian telomeres. Oncogene 2002;21:532–540.
13 Chen FM: Sr2+ facilitates intermolecular G-quadruplex formation of telomeric sequences. Biochemistry 1992;31:3769–3776.
14 Wang Y, Patel DJ: Guanine residues in d(T2AG3) and d(T2G4) form parallel-stranded potassium cation stabilized G-quadruplexes with anti glycosidic torsion angles in solution. Biochemistry 1992;31:8112–8119.
15 Karlseder J, Hoke K, Mirzoeva OK, Bakkenist C, Kastan MB, Petrini JH, de Lange T: The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. PLoS Biol 2004;2:E240.
16 Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW: Telomerase activity in human germline and embryonic tissues and cells. Dev Genet 1996;18:173–179.

17 Schaetzlein S, Lucas-Hahn A, Lemme E, Kues WA, Dorsch M, Manns MP, Niemann H, Rudolph KL: Telomere length is reset during early mammalian embryogenesis. Proc Natl Acad Sci USA 2004;101:8034–8038.
18 Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB: Telomere end-replication problem and cell aging. J Mol Biol 1992;225:951–960.
19 Sfeir AJ, Chai W, Shay JW, Wright WE: Telomere-end processing the terminal nucleotides of human chromosomes. Mol Cell 2005;18:131–138.
20 Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB: Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci USA 1992;89:10114–10118.
21 Zou Y, Sfeir A, Gryaznov SM, Shay JW, Wright WE: Does a sentinel or a subset of short telomeres determine replicative senescence? Mol Biol Cell 2004;15:3709–3718.
22 Shay JW, Wright WE: Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis 2005;26:867–874.
23 Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279:349–352.
24 Xiaoxue Y, Zhongqiang C, Zhaoqing G, Gengting D, Qingjun M, Shenwu W: Immortalization of human osteoblasts by transferring human telomerase reverse transcriptase gene. Biochem Biophys Res Commun 2004;315:643–651.
25 Wege H, Le HT, Chui MS, Liu L, Wu J, Giri R, Malhi H, Sappal BS, Kumaran V, Gupta S, Zern MA: Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. Gastroenterology 2003;124:432–444.
26 Bai Y, Hu Q, Li X, Wang Y, Lin C, Shen L, Li L: Telomerase immortalization of human neural progenitor cells. Neuroreport 2004;15:245–249.
27 Chin L, Artandi SE, Shen Q, Tam A, Lee SL, Gottlieb GJ, Greider CW, DePinho RA: p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell 1999;97:527–538.
28 Wong KK, Maser RS, Bachoo RM, Menon J, Carrasco DR, Gu Y, Alt FW, DePinho RA: Telomere dysfunction and Atm deficiency compromises organ homeostasis and accelerates ageing. Nature 2003;421:643–648.
29 Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM: Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). Mol Cell 2004;14:501–513.
30 Satyanarayana A, Greenberg RA, Schaetzlein S, Buer J, Masutomi K, Hahn WC, Zimmermann S, Martens U, Manns MP, Rudolph KL: Mitogen stimulation cooperates with telomere shortening to activate DNA damage responses and senescence signaling. Mol Cell Biol 2004;24:5459–5474.
31 d'Adda dF, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, von Zglinicki T, Saretzki G, Carter NP, Jackson SP: A DNA damage checkpoint response in telomere-initiated senescence. Nature 2003;426:194–198.
32 Bodnar AG, Kim NW, Effros RB, Chiu CP: Mechanism of telomerase induction during T cell activation. Exp Cell Res 1996;228:58–64.
33 Martens UM, Brass V, Sedlacek L, Pantic M, Exner C, Guo Y, Engelhardt M, Lansdorp PM, Waller CF, Lange W: Telomere maintenance in human B lymphocytes. Br J Haematol 2002;119:810–818.
34 Chiu CP, Dragowska W, Kim NW, Vaziri H, Yui J, Thomas TE, Harley CB, Lansdorp PM: Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. Stem Cells 1996;14:239–248.
35 Ulaner GA, Hu JF, Vu TH, Giudice LC, Hoffman AR: Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. Cancer Res 1998;58:4168–4172.
36 Ulaner GA, Hu JF, Vu TH, Giudice LC, Hoffman AR: Tissue-specific alternate splicing of human telomerase reverse transcriptase (hTERT) influences telomere lengths during human development. Int J Cancer 2001;91:644–649.

37 Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura H, Nakanishi T, Tahara E, Ide T, Ishikawa F: Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. Nat Genet 1998;18:65–68.
38 Masutomi K, Yu EY, Khurts S, Ben Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC: Telomerase maintains telomere structure in normal human cells. Cell 2003;114:241–253.
39 Inui T, Shinomiya N, Fukasawa M, Kobayashi M, Kuranaga N, Ohkura S, Seki S: Growth-related signaling regulates activation of telomerase in regenerating hepatocytes. Exp Cell Res 2002;273:147–156.
40 Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, DePinho RA: Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell 1999;96:701–712.
41 Herrera E, Samper E, Martin-Caballero J, Flores JM, Lee HW, Blasco MA: Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. EMBO J 1999;18:2950–2960.
42 Lee HW, Blasco MA, Gottlieb GJ, Horner JW, Greider CW, DePinho RA: Essential role of mouse telomerase in highly proliferative organs. Nature 1998;392:569–574.
43 Wong KK, Chang S, Weiler SR, Ganesan S, Chaudhuri J, Zhu C, Artandi SE, Rudolph KL, Gottlieb GJ, Chin L, Alt FW, DePinho RA: Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. Nat Genet 2000;26:85–88.
44 Rudolph KL, Millard M, Bosenberg MW, DePinho RA: Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat Genet 2001;28:155–159.
45 Satyanarayana A, Wiemann SU, Buer J, Lauber J, Dittmar KE, Wustefeld T, Blasco MA, Manns MP, Rudolph KL: Telomere shortening impairs organ regeneration by inhibiting cell cycle re-entry of a subpopulation of cells. EMBO J 2003;22:4003–4013.
46 Gonzalez-Suarez E, Samper E, Ramirez A, Flores JM, Martin-Caballero J, Jorcano JL, Blasco MA: Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. EMBO J 2001;20:2619–2630.
47 Gonzalez-Suarez E, Geserick C, Flores JM, Blasco MA: Antagonistic effects of telomerase on cancer and aging in K5-mTert transgenic mice. Oncogene 2005;24:2256–2270.
48 Harle-Bachor C, Boukamp P: Telomerase activity in the regenerative basal layer of the epidermis in human skin and in immortal and carcinoma-derived skin keratinocytes. Proc Natl Acad Sci USA 1996;93:6476–6481.
49 Broccoli D, Young JW, de Lange T: Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci USA 1995;92:9082–9086.
50 Yui J, Chiu CP, Lansdorp PM: Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. Blood 1998;91:3255–3262.
51 Fajkus J, Borsky M, Kunicka Z, Kovarikova M, Dvorakova D, Hofmanova J, Kozubik A: Changes in telomerase activity, expression and splicing in response to differentiation of normal and carcinoma colon cells. Anticancer Res 2003;23:1605–1612.
52 Ostenfeld T, Caldwell MA, Prowse KR, Linskens MH, Jauniaux E, Svendsen CN: Human neural precursor cells express low levels of telomerase in vitro and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. Exp Neurol 2000;164:215–226.
53 Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P: Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci USA 2003;100:10440–10445.
54 Zimmermann S, Voss M, Kaiser S, Kapp U, Waller CF, Martens UM: Lack of telomerase activity in human mesenchymal stem cells. Leukemia 2003;17:1146–1149.
55 Parsch D, Fellenberg J, Brummendorf TH, Eschlbeck AM, Richter W: Telomere length and telomerase activity during expansion and differentiation of human mesenchymal stem cells and chondrocytes. J Mol Med 2004;82:49–55.
56 Morrison SJ, Prowse KR, Ho P, Weissman IL: Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity 1996;5:207–216.
57 Djojosubroto MW, Choi YS, Lee HW, Rudolph KL: Telomeres and telomerase in aging, regeneration and cancer. Mol Cells 2003;15:164–175.

58 Zimmermann S, Martens UM: Telomere dynamics in hematopoietic stem cells. Curr Mol Med 2005;5:179–185.
59 Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM: Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. Proc Natl Acad Sci USA 1994;91:9857–9860.
60 Robertson JD, Testa NG, Russell NH, Jackson G, Parker AN, Milligan DW, Stainer C, Chakrabarti S, Dougal M, Chopra R: Accelerated telomere shortening following allogeneic transplantation is independent of the cell source and occurs within the first year post transplant. Bone Marrow Transplant 2001;27:1283–1286.
61 Rufer N, Brummendorf TH, Chapuis B, Helg C, Lansdorp PM, Roosnek E: Accelerated telomere shortening in hematological lineages is limited to the first year following stem cell transplantation. Blood 2001;97:575–577.
62 Ross EA, Anderson N, Micklem HS: Serial depletion and regeneration of the murine hematopoietic system. Implications for hematopoietic organization and the study of cellular aging. J Exp Med 1982;155:432–444.
63 Chen J: Senescence and functional failure in hematopoietic stem cells. Exp Hematol 2004;32: 1025–1032.
64 Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA: Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 2005;433:760–764.
65 Allsopp RC, Morin GB, DePinho R, Harley CB, Weissman IL: Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. Blood 2003;102:517–520.
66 Samper E, Fernandez P, Eguia R, Martin-Rivera L, Bernad A, Blasco MA, Aracil M: Long-term repopulating ability of telomerase-deficient murine hematopoietic stem cells. Blood 2002;99: 2767–2775.
67 Allsopp RC, Morin GB, Horner JW, DePinho R, Harley CB, Weissman IL: Effect of TERT overexpression on the long-term transplantation capacity of hematopoietic stem cells. Nat Med 2003;9:369–371.
68 Hemann MT, Rudolph KL, Strong MA, DePinho RA, Chin L, Greider CW: Telomere dysfunction triggers developmentally regulated germ cell apoptosis. Mol Biol Cell 2001;12:2023–2030.
69 Flores I, Cayuela ML, Blasco MA: Effects of telomerase and telomere length on epidermal stem cell behavior. Science 2005;309:1253–1256.
70 Liu L, DiGirolamo CM, Navarro PA, Blasco MA, Keefe DL: Telomerase deficiency impairs differentiation of mesenchymal stem cells. Exp Cell Res 2004;294:1–8.
71 Armstrong L, Saretzki G, Peters H, Wappler I, Evans J, Hole N, von Zglinicki T, Lako M: Overexpression of telomerase confers growth advantage, stress resistance, and enhanced differentiation of ESCs toward the hematopoietic lineage. Stem Cells 2005;23:516–529.
72 Sarin KY, Cheung PT, Gilison D, Lee E, Tennen RI, Wang E, Artandi MK, Oro AE, Artandi SE: Conditional telomerase induction causes proliferation of hair follicle stem cells. Nature 2005;436:1048–1052.
73 Hong Y, Stambrook PJ: Restoration of an absent G1 arrest and protection from apoptosis in embryonic stem cells after ionizing radiation. Proc Natl Acad Sci USA 2004;101:14443–14448.
74 Aladjem MI, Spike BT, Rodewald LW, Hope TJ, Klemm M, Jaenisch R, Wahl GM: ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. Curr Biol 1998;8:145–155.
75 Puri PL, Bhakta K, Wood LD, Costanzo A, Zhu J, Wang JY: A myogenic differentiation checkpoint activated by genotoxic stress. Nat Genet 2002;32:585–593.
76 Ferron S, Mira H, Franco S, Cano-Jaimez M, Bellmunt E, Ramirez C, Farinas I, Blasco MA: Telomere shortening and chromosomal instability abrogates proliferation of adult but not embryonic neural stem cells. Development 2004;131:4059–4070.
77 Mitchell JR, Wood E, Collins K: A telomerase component is defective in the human disease dyskeratosis congenita. Nature 1999;402:551–555.
78 Mason PJ, Wilson DB, Bessler M: Dyskeratosis congenita – a disease of dysfunctional telomere maintenance. Curr Mol Med 2005;5:159–170.
79 Dokal I: Dyskeratosis congenita. Br J Haematol 1999;105(suppl 1):11–15.

80 Vulliamy T, Marrone A, Goldman F, Dearlove A, Bessler M, Mason PJ, Dokal I: The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. Nature 2001;413: 432–435.
81 Vulliamy T, Marrone A, Szydlo R, Walne A, Mason PJ, Dokal I: Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC. Nat Genet 2004;36:447–449.
82 Knudson M, Kulkarni S, Ballas ZK, Bessler M, Goldman F: Association of immune abnormalities with telomere shortening in autosomal-dominant dyskeratosis congenita. Blood 2005;105: 682–688.
83 Yamaguchi H, Baerlocher GM, Lansdorp PM, Chanock SJ, Nunez O, Sloand E, Young NS: Mutations of the human telomerase RNA gene (TERC) in aplastic anemia and myelodysplastic syndrome. Blood 2003;102:916–918.
84 Hanson H, Mathew CG, Docherty Z, Mackie OC: Telomere shortening in Fanconi anaemia demonstrated by a direct FISH approach. Cytogenet Cell Genet 2001;93:203–206.
85 Leteurtre F, Li X, Guardiola P, Le Roux G, Sergere JC, Richard P, Carosella ED, Gluckman E: Accelerated telomere shortening and telomerase activation in Fanconi's anaemia. Br J Haematol 1999;105:883–893.
86 Yamaguchi H, Calado RT, Ly H, Kajigaya S, Baerlocher GM, Chanock SJ, Lansdorp PM, Young NS: Mutations in *TERT*, the gene for telomerase reverse transcriptase, in aplastic anemia. N Engl J Med 2005;352:1413–1424.
87 Ball SE, Gibson FM, Rizzo S, Tooze JA, Marsh JC, Gordon-Smith EC: Progressive telomere shortening in aplastic anemia. Blood 1998;91:3582–3592.
88 Brummendorf TH, Maciejewski JP, Mak J, Young NS, Lansdorp PM: Telomere length in leukocyte subpopulations of patients with aplastic anemia. Blood 2001;97:895–900.
89 Hande MP, Samper E, Lansdorp P, Blasco MA: Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. J Cell Biol 1999;144:589–601.
90 d'Adda dF, Hande MP, Tong WM, Lansdorp PM, Wang ZQ, Jackson SP: Functions of poly(ADP-ribose) polymerase in controlling telomere length and chromosomal stability. Nat Genet 1999;23: 76–80.
91 Hackett JA, Feldser DM, Greider CW: Telomere dysfunction increases mutation rate and genomic instability. Cell 2001;106:275–286.
92 Kirk KE, Harmon BP, Reichardt IK, Sedat JW, Blackburn EH: Block in anaphase chromosome separation caused by a telomerase template mutation. Science 1997;275:1478–1481.
93 Greenberg RA, Chin L, Femino A, Lee KH, Gottlieb GJ, Singer RH, Greider CW, DePinho RA: Short dysfunctional telomeres impair tumorigenesis in the INK4a(delta2/3) cancer-prone mouse. Cell 1999;97:515–525.
94 Gonzalez-Suarez E, Samper E, Flores JM, Blasco MA: Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. Nat Genet 2000;26:114–117.
95 Farazi PA, Glickman J, Jiang S, Yu A, Rudolph KL, DePinho RA: Differential impact of telomere dysfunction on initiation and progression of hepatocellular carcinoma. Cancer Res 2003;63:5021–5027.
96 Maser RS, DePinho RA: Connecting chromosomes, crisis, and cancer. Science 2002;297: 565–569.
97 Satyanarayana A, Manns MP, Rudolph KL: Telomeres and telomerase: a dual role in hepatocarcinogenesis. Hepatology 2004;40:276–283.
98 Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, DePinho RA: Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 2000;406:641–645.
99 Franco S, Segura I, Riese HH, Blasco MA: Decreased B16F10 melanoma growth and impaired vascularization in telomerase-deficient mice with critically short telomeres. Cancer Res 2002;62: 552–559.
100 Blasco MA: Immunosenescence phenotypes in the telomerase knockout mouse. Springer Semin Immunopathol 2002;24:75–85.
101 Neuber K, Schmidt S, Mensch A: Telomere length measurement and determination of immunosenescence-related markers (CD28, CD45RO, CD45RA, interferon-gamma and interleukin-4) in skin-homing T cells expressing the cutaneous lymphocyte antigen: indication of a non-ageing T-cell subset. Immunology 2003;109:24–31.

102 Artandi SE, Alson S, Tietze MK, Sharpless NE, Ye S, Greenberg RA, Castrillon DH, Horner JW, Weiler SR, Carrasco RD, DePinho RA: Constitutive telomerase expression promotes mammary carcinomas in aging mice. Proc Natl Acad Sci USA 2002;99:8191–8196.
103 Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA: Creation of human tumour cells with defined genetic elements. Nature 1999;400:464–468.
104 Seger YR, Garcia-Cao M, Piccinin S, Cunsolo CL, Doglioni C, Blasco MA, Hannon GJ, Maestro R: Transformation of normal human cells in the absence of telomerase activation. Cancer Cell 2002;2:401–413.
105 Lazarov M, Kubo Y, Cai T, Dajee M, Tarutani M, Lin Q, Fang M, Tao S, Green CL, Khavari PA: CDK4 coexpression with Ras generates malignant human epidermal tumorigenesis. Nat Med 2002;8:1105–1114.
106 Stewart SA, Hahn WC, O'Connor BF, Banner EN, Lundberg AS, Modha P, Mizuno H, Brooks MW, Fleming M, Zimonjic DB, Popescu NC, Weinberg RA: Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. Proc Natl Acad Sci USA 2002;99:12606–12611.
107 Li S, Rosenberg JE, Donjacour AA, Botchkina IL, Hom YK, Cunha GR, Blackburn EH: Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. Cancer Res 2004;64:4833–4840.
108 Stewart SA, Ben Porath I, Carey VJ, O'Connor BF, Hahn WC, Weinberg RA: Erosion of the telomeric single-strand overhang at replicative senescence. Nat Genet 2003;33:492–496.
109 Li S, Crothers J, Haqq CM, Blackburn EH: Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. J Biol Chem 2005;280:23709–23717.
110 Smith LL, Coller HA, Roberts JM: Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat Cell Biol 2003;5:474–479.
111 Cayuela ML, Flores JM, Blasco MA: The telomerase RNA component Terc is required for the tumour-promoting effects of Tert overexpression. EMBO Rep 2005;6:268–274.
112 Ohyashiki JH, Sashida G, Tauchi T, Ohyashiki K: Telomeres and telomerase in hematologic neoplasia. Oncogene 2002;21:680–687.
113 Engelhardt M, Mackenzie K, Drullinsky P, Silver RT, Moore MA: Telomerase activity and telomere length in acute and chronic leukemia, pre- and post-ex vivo culture. Cancer Res 2000;60: 610–617.
114 Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S: Telomerase activity in normal leukocytes and in hematologic malignancies. Blood 1995;85:2315–2320.
115 Trentin L, Ballon G, Ometto L, Perin A, Basso U, Chieco-Bianchi L, Semenzato G, De Rossi A: Telomerase activity in chronic lymphoproliferative disorders of B-cell lineage. Br J Haematol 1999;106:662–668.
116 Bechter OE, Eisterer W, Pall G, Hilbe W, Kuhr T, Thaler J: Telomere length and telomerase activity predict survival in patients with B cell chronic lymphocytic leukemia. Cancer Res 1998;58: 4918–4922.
117 Damle RN, Batliwalla FM, Ghiotto F, Valetto A, Albesiano E, Sison C, Allen SL, Kolitz J, Vinciguerra VP, Kudalkar P, Wasil T, Rai KR, Ferrarini M, Gregersen PK, Chiorazzi N: Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations. Blood 2004;103:375–382.
118 Brousset P, Al Saati T, Chaouche N, Zenou RC, Schlaifer D, Chittal S, Delsol G: Telomerase activity in reactive and neoplastic lymphoid tissues: infrequent detection of activity in Hodgkin's disease. Blood 1997;89:26–31.
119 Ely SA, Chadburn A, Dayton CM, Cesarman E, Knowles DM: Telomerase activity in B-cell non-Hodgkin lymphoma. Cancer 2000;89:445–452.
120 Norrback KF, Dahlenborg K, Carlsson R, Roos G: Telomerase activation in normal B lymphocytes and non-Hodgkin's lymphomas. Blood 1996;88:222–229.
121 Norrback KF, Enblad G, Erlanson M, Sundstrom C, Roos G: Telomerase activity in Hodgkin's disease. Blood 1998;92:567–573.
122 Ohyashiki K, Ohyashiki JH, Iwama H, Hayashi S, Shay J W, Toyama K: Telomerase activity and cytogenetic changes in chronic myeloid leukemia with disease progression. Leukemia 1997;11: 190–194.

123 Ohyashiki K, Iwama H, Yahata N, Tauchi T, Kawakubo K, Shimamoto T, Ohyashiki JH: Telomere dynamics in myelodysplastic syndromes and acute leukemic transformation. Leuk Lymphoma 2001;42:291–299.
124 Ladetto M, Compagno M, Ricca I, Pagano M, Rocci A, Astolfi M, Drandi D, di Celle PF, Dell'aquila M, Mantoan B, Vallet S, Pagliano G, De Marco F, Francese R, Santo L, Cuttica A, Marinone C, Boccadoro M, Tarella C: Telomere length correlates with histopathogenesis according to the germinal center in mature B-cell lymphoproliferative disorders. Blood 2004;103:4644–4649.
125 Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, Sanz M, Vallespi T, Hamblin T, Oscier D, Ohyashiki K, Toyama K, Aul C, Mufti G, Bennett J: International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89:2079–2088.
126 Sieglova Z, Zilovcova S, Cermak J, Rihova H, Brezinova D, Dvorakova R, Markova M, Maaloufova J, Sajdova J, Brezinova J, Zemanova Z, Michalova K: Dynamics of telomere erosion and its association with genome instability in myelodysplastic syndromes (MDS) and acute myelogenous leukemia arising from MDS: a marker of disease prognosis? Leuk Res 2004;28: 1013–1021.
127 Ohyashiki JH, Ohyashiki K, Fujimura T, Kawakubo K, Shimamoto T, Iwabuchi A, Toyama K: Telomere shortening associated with disease evolution patterns in myelodysplastic syndromes. Cancer Res 1994;54:3557–3560.
128 Ohyashiki JH, Iwama H, Yahata N, Ando K, Hayashi S, Shay JW, Ohyashiki K: Telomere stability is frequently impaired in high-risk groups of patients with myelodysplastic syndromes. Clin Cancer Res 1999;5:1155–1160.
129 Brummendorf TH, Holyoake TL, Rufer N, Barnett MJ, Schulzer M, Eaves CJ, Eaves AC, Lansdorp PM: Prognostic implications of differences in telomere length between normal and malignant cells from patients with chronic myeloid leukemia measured by flow cytometry. Blood 2000;95:1883–1890.
130 Iwama H, Ohyashiki K, Ohyashiki JH, Hayashi S, Kawakubo K, Shay JW, Toyama K: The relationship between telomere length and therapy-associated cytogenetic responses in patients with chronic myeloid leukemia. Cancer 1997;79:1552–1560.
131 Boultwood J, Peniket A, Watkins F, Shepherd P, McGale P, Richards S, Fidler C, Littlewood TJ, Wainscoat JS: Telomere length shortening in chronic myelogenous leukemia is associated with reduced time to accelerated phase. Blood 2000;96:358–361.
132 Brummendorf TH, Rufer N, Holyoake TL, Maciejewski J, Barnett MJ, Eaves CJ, Eaves AC, Young N, Lansdorp PM: Telomere length dynamics in normal individuals and in patients with hematopoietic stem cell-associated disorders. Ann N Y Acad Sci 2001;938:293–303.
133 Lee JJ, Nam CE, Cho SH, Park KS, Chung IJ, Kim HJ: Telomere length shortening in non-Hodgkin's lymphoma patients undergoing chemotherapy. Ann Hematol 2003;82:492–495.
134 Yamada K, Yajima T, Yagihashi A, Kobayashi D, Koyanagi Y, Asanuma K, Yamada M, Moriai R, Kameshima H, Watanabe N: Role of human telomerase reverse transcriptase and telomeric-repeat binding factor proteins 1 and 2 in human hematopoietic cells. Jpn J Cancer Res 2000;91:1278–1284.
135 Yamada K, Yagihashi A, Yamada M, Asanuma K, Moriai R, Kobayashi D, Tsuji N, Watanabe N: Decreased gene expression for telomeric-repeat binding factors and TIN2 in malignant hematopoietic cells. Anticancer Res 2002;22:1315–1320.

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Retrotransposable Elements and Human Disease

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Abstract

Nearly 50% of the human genome is composed of fossils from the remains of past transposable element duplication. Mobilization continues in the genomes of extant humans but is now restricted to retrotransposons, a class of mobile elements that move via a copy and paste mechanism. Currently active retrotransposable elements include Long INterspersed Elements (LINEs), Short INterspersed Elements (SINEs) and SVA (SINE/VNTR/*Alu*) elements. Retrotransposons are responsible for creating genetic variation and on occasion, disease-causing mutations, within the human genome. Approximately 0.27% of all human disease mutations are attributable to retrotransposable elements. Different mechanisms of genome alteration created by retrotransposable elements include insertional mutagenesis, recombination, retrotransposition-mediated and gene conversion-mediated deletion, and 3′ transduction. Although researchers in the field of human genetics have discovered many mutational mechanisms for retrotransposable elements, their contribution to genetic variation within humans is still being resolved.

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Transposable Elements in the Human Genome

Almost the entire human genome is ubiquitously littered with the skeletons of mobile elements, which all told, account for a staggering 45% of the sequence content [1]. Mobile elements successfully accumulated in genomes during eukaryotic evolution and are grouped into one of two different classes: DNA transposons or retrotransposons.

DNA transposons constitute 3% of the human genome [1] and although they are represented by inactive fossils in humans, DNA transposons remain

active in the genomes of plants, flies and bacteria [2–4]. Retrotransposons, on the other hand, are currently actively mobilizing within the human genome and comprise approximately 40% of the DNA sequence [1]. Due to the current propagation of retrotransposons in humans, they will be the focus of this review.

Retrotransposons, by definition, mobilize via an RNA intermediate that is subsequently reverse transcribed into a cDNA copy using a mechanism termed Target Primed Reverse Transcription (TPRT) [5]. This copy and paste mechanism of mobilization results in the spread of retrotransposons to new genomic locations. Retrotransposable elements are categorized based on their ability to mobilize. Long INterspersed Elements (LINEs) are autonomous retrotransposons that encode the enzymatic machinery required for their propagation [6]. Short INterspersed Elements (SINEs), such as *Alu*, and SVA (SINE/VNTR/*Alu*) elements, are non-autonomous and thus require the enzymatic machinery of LINE elements for retrotransposition [7, 8].

Over the last quarter century, many ideas concerning the function of mobile elements have been put forth. Orgel and Crick were proponents of the idea that mobile elements served no function and resided as parasitic entities within the genome, without contributing to the evolutionary well-being of the organism [9]. Others have hypothesized that mobile elements function as origins of replication [10], chromosomal band-aids [11] and mediators of translational activation [12].

Despite disagreement over the function of mobile elements, they constitute an interesting source of human genomic variation and occasionally, disease. Here we present an overview of the contribution of mobile elements, in particular, retrotransposable elements, to genetic disease in *Homo sapiens.*

Autonomous Retrotransposons and Disease

Long INterspersed Elements

Computational analyses of the human genome have shown that L1 elements have reached a copy number in excess of 500,000 and comprise some 17% of the genomic sequence [1]. Numerous studies indicate that some subclasses of L1 element are still actively expanding by retrotransposition in extant human genomes [6]. Retrotranspositionally active L1 elements are approximately 6 kb in length, as shown in figure 1a. Evidence suggests that L1 elements have orchestrated large-scale alterations in the genomic architecture of human beings, as they are the major source of reverse transcriptase, upon which other retrotransposable elements and processed pseudogenes have amplified [6]. As a result, L1 elements are both directly and indirectly responsible for the vast

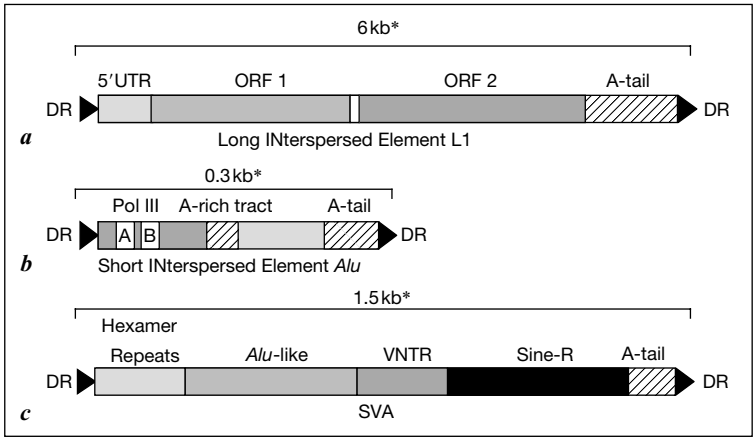

Fig. 1. Active retrotransposons within the human genome. ***a*** Long INterspersed Element L1. L1s are approximately 6 kb long and possess a 5′ UTR, within which resides an RNA polymerase II promoter. Full-length elements encode two open reading frames that produce a reverse transcriptase and endonuclease, as well as an RNA binding protein. Each L1 element has a 3′ UTR, an oligo-dA tail and is flanked by direct repeat sequences (DR). ***b*** Short INterspersed Element *Alu*. *Alu* SINEs are approximately 300 bp long and comprise two arms separated by a middle A-rich tract. They possess an RNA polymerase III (POL III) promoter (A and B box), in addition to a variable length oligo-dA rich tail. *Alu* elements are flanked by short direct repeats (DR). ***c*** SVA. Full-length SVA elements are approximately 1.5 kb long, and are composed of several repeat elements: a $CCCTCT_n$ hexamer repeat, an anti-sense *Alu*, a variable number of tandem repeats, and a SINE-R element. SVA elements possess an oligo dA-rich tail and are flanked by short direct repeats (DR).* Not drawn to scale.

majority of retrotransposable element-derived variation and disease within the human genome. The propagation of L1 has resulted in disease-causing de novo insertions within genes, many of which disrupt exons or alter RNA splicing in the mutant alleles. In addition, the 500,000 L1 elements in the human genome provide long regions of sequence identity that represent numerous sites for unequal homologous recombination and mutation. Despite their vast numbers and retrotransposition activity, L1 elements are directly responsible for less than 20% of all retrotransposable element-related human diseases, even though experimental evidence suggests that L1s demonstrate a *cis* preference for their own replication machinery (see review [6]). The paucity of disease-causing L1 insertions may stem from L1 AT-rich insertion preference, essentially sidestepping the sensitive coding regions of the genome, or perhaps new L1 insertions are subject to appreciable amounts of negative selection because of their size. Additionally, distant L1 spacing may mean that recombination between L1

elements would induce fatal genetic damage and be eliminated. Due to the paucity of disease-causing L1 recombination events, we will not cover this particular mechanism here. Instead, we will focus on what is currently known concerning L1 retrotransposition, retrotransposition-mediated genomic deletion and 3′ transduction and their contribution to human diseases.

L1 Retrotransposition

Newly inserted L1 elements have induced disease in sixteen separate documented cases and the vast majority of these elements belong to one of the youngest L1 subfamilies, termed Ta. The L1 Ta subfamily is approximately 2 million years old and shows a high level of polymorphism (insertion presence/absence) in diverse human populations [13].

In 2001, a comprehensive study of newly inserted L1 elements and related diseases was published [6]. The data gathered in this study indicated that nine out of the thirteen disease-causing L1 insertions discovered until that time disrupted sex-linked genes, namely Factor VIII, dystrophin or *CYBB* [6]. This observation suggests that some genes are hotspots for mobile element integration, or that the ensuing genic damage was easily detected due to their genomic position on the X chromosome, i.e. through ascertainment bias. Since the review in 2001 [6], three new cases of L1 induced X-linked genetic disease have been discovered. The first case describes an L1 insertion into the *RPS6KA3* gene causing Coffin-Lowry syndrome [14]. Second, a disruption of intronic splicing through an L1 insertion into the *CHM* gene causing choroideremia [15], and finally, a case of hemophilia B induced by L1 disruption of the Factor IX gene [16].

L1 disease-causing insertions have been mapped to both the exons and introns of genes. Most exonic L1 integrations are presumably lethal due to the introduction of premature stop codons and are likely eliminated from the population. However, nine instances of exonic integration have resulted in phenotypically tolerable diseases in humans. Intronic L1 insertions may also be lethal, but some studies have documented the existence of tolerable intronic insertions [6]. L1 elements have recently been shown to reduce mRNA transcript levels due to their presence within introns [17]. This phenomenon is related to the inefficiency of RNA polymerase II to transcribe through L1 elements [17]. Researchers suggest that L1 elements may act as ‘molecular rheostats’ by directly altering gene expression in this way [17]. Another study also recently demonstrated that RNA polymerase II transcription of L1 elements is adversely affected due to multiple termination and polyadenylation signals along the length of the L1 element [18]. It was proposed that premature RNA polymerase II termination could be a way that L1 elements limit their damage to host genomes [18]. At the same time, it would also mean that the stalling of polymerase molecules along L1 sequence would increase the negative impact of L1

insertions into genes [18]. Intergenic insertions of L1 may also alter gene expression throughout the human genome. L1 elements possess one RNA polymerase II promoter on their sense strand and another on their anti-sense strand that have been implicated in the enhancement of some genes (Factor IX and apolipoprotein A genes) and in the formation of chimeric mRNA transcripts [6]. Given the high insertion polymorphism levels of young L1 elements within the human genome, intronic and intergenic insertions could profoundly influence gene expression on both the individual and population level.

L1 Retrotransposition-Mediated Deletion

L1 retrotransposition-mediated deletion was first reported in 2002, where L1 integrations within cultured human cells resulted in target site deletions spanning from 1 bp to 70,000 bp at a rate of about 10% [19–21]. These studies hinted at the vast impact that L1 retrotransposition-mediated deletion may have had on primate genomes. If 10% of the L1 retrotranspositions induced deletions, then over 5,000 L1 retrotranspositions would be responsible for eliminating megabases of primate genomic DNA.

Retrotransposition events that resulted in deleted target site DNA were found to possess atypical characteristics, including a lack of target site duplications (TSDs), non-canonical L1 EN (endonuclease) nick sites and sometimes the absence of an oligo-dA rich tail (see [11, 20, 21]). Researchers proposed two models, based on evidence from in vitro retrotransposition studies, to help explain the mechanism for the insertion-deletion events. The first model proposed that L1 EN nicking variation on the top strand could account for TSD-less L1 element structure, in addition to genomic deletion at the site of insertion [11, 20, 21]. The second mechanism suggested that L1 reverse transcriptase could initiate TPRT from existing breaks in the genome, not depending on L1 EN for the initial nick [11]. Recently, a third model was formulated to explain the mechanism of retrotransposition-mediated deletion, named promiscuous TPRT (pTPRT) [22]. This model states that a retrotransposable element RNA transcript may hybridize to a region of genomic DNA downstream of a genomic break in order to initiate TPRT. The displaced single stranded DNA is removed through enzymatic degradation or by mechanical force, in order to create the target site deletion.

A recent survey of L1 disease-causing insertions reported two instances of retrotransposition-mediated deletion in humans: a 1-bp deletion in the *DMD* gene and another 6-bp deletion in the *FCMD* gene that resulted in Duchenne muscular dystrophy and Fukuyama-type congenital muscular dystrophy, respectively [23, 24]. In both cases, the disease phenotype resulted from the L1 element insertion, rather than through deletion of genomic sequence at the target site. These two cases are among only six other published in vivo examples

of L1 retrotransposition-mediated deletion in the human genome to date [25, 26]. Further research is underway at this time to determine the frequency of L1 retrotransposition-mediated deletion in the native human genome and its resultant impact on genomic instability and evolution.

L1-Mediated 3′ Transduction

A decade ago, a mechanism was detected by which L1 alters the primate genome. It was termed 3′ transduction [27]. The discovery of 3′ transduction coincided with the insertion of L1 into the dystrophin gene, manifesting muscular dystrophy in a single human individual [27]. Since then, cell based studies have documented the ability of L1 elements to shuffle genomic DNA, including exons, using this mechanism (see [28]). During 3′ transduction, a read-through transcript of the L1 element transcribes flanking genomic material downstream by virtue of a weak L1 termination and polyadenylation signal. Transduction of adjacent genomic DNA by L1 elements may result in the creation of new exons and in the alteration of gene expression through promoter and enhancer shuffling. Computational analyses have indicated that L1-mediated transduction of genomic material may occur at a rate of one in every five L1 retrotransposition events and that approximately 1% of the human haploid genome may have arisen by this mechanism [29]. In some instances, due to the severe truncation of L1 elements upon reverse transcription, it is possible that the transduced sequence will not reside adjacent to its L1 element thereby artificially reducing estimates of the impact that 3′ transduction has had on the architecture of the human genome.

Non-Autonomous Retrotransposons and Disease

Alu Elements

The *Alu* family represents an enormously successful lineage of retrotransposons, whose origin and amplification coincided with the radiation of primates some 65 million years ago [5]. *Alu* elements are non-autonomous retrotransposons that mobilize in a copy and paste fashion. They are approximately 300 bp long and comprise two nearly identical arms separated by a middle A-rich tract, in addition to a 3′ oligo dA-rich tail (fig. 1b). Recent data suggest that only a fraction of *Alu* elements, termed source genes, are retrotranspositionally competent and responsible for producing over one million *Alu* copies within the primate order [5]. Although the exact characteristics of a source gene are unclear, *Alu* element age, RNA polymerase III promoter integrity and the length and homogeneity of the oligo-dA rich tail are considered major factors influencing retrotransposition potential [5]. *Alu* elements have continued to mobilize throughout the evolution of primates, as evidenced by human lineage-specific elements.

These elements are absent from orthologous loci in non-human primates and exhibit high levels of polymorphism with respect to their insertion presence and absence in different human individuals. Recent estimates of *Alu* insertion numbers in the human lineage (~7000–9000) suggest that *Alu* elements are amplifying at a rate of one new insert approximately every 15–20 births (see [30] for theory). Thus, it is not surprising that recent *Alu* retrotransposition events have given rise to a number of human diseases.

Alu elements are known to create genetic instability and disease in a number of different ways. We will deal with each mechanism in turn and assess the prevalence, importance and resultant impact on the integrity of the human genome.

Alu Retrotransposition

From a review of current literature, 25 newly integrated *Alu* elements have been determined to induce disease states in human beings. Approximately eleven of the *Alu* elements integrated within introns and either caused partial intron retention within the mature mRNA through *Alu* exonization, or exon skipping [6, 31–36]. A study by Lev-Maor et al. described the process of *Alu* exonization in a 2003 study, where the retention of anti-sense *Alu* elements within the mature mRNA transcript was attributed to the introduction of new splice sites from the *Alu* sequence [32]. One recent study has proposed that exonized *Alu* elements are almost exclusively alternatively spliced, and that '*Alu*ternative' splicing is accountable for producing variable exonic transcripts in over 5% of genes [37]. The retention of *Alu* elements within mRNA transcripts could contribute to subtle differences in gene expression between individuals and populations.

Alu repeats are rarely found within the coding regions of genes, as this may disrupt the gene's function. However, twelve exon insertion events have been described in the literature (see review [6]). Since the publication of that review in 2001, two other studies have reported *Alu* integration into exons as the cause of genetic disease. In the first case, a young *Alu*Ya5 element inserted into codon 650 of the renal chloride channel gene, *CLCN5*, resulting in Dent's disease, a cause of renal failure [38]. The second study reports a case of hemophilia A as a direct result of *Alu* integration into exon 14 of the Factor VIII gene [39]. The total number of *Alu* retrotransposition insertions (both intronic and exonic) contributing to disease phenotypes within the human lineage equals 25. The total number of mutations in the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html) currently exceeds 44,000, as of January 2005. Therefore, *Alu* element insertional disruption accounts for 0.05% of all human mutations. However, only non-lethal mutations that cause observable phenotypes will be captured by this statistic. *Alu* insertions that are lethal and those that cause only mild phenotypes will be missed and thereby underestimate the true number of detrimental *Alu* insertions.

Alu-Alu Recombination

Alu-Alu unequal homologous recombination usually involves crossover between evolutionarily older elements within the genome (see [40]). *Alu* elements appear to possess particular characteristics that make them prone to recombination. These are: (1) the relatively close proximity of *Alu* elements within the genome, making most recombination events tolerable. (2) The sequence identity of *Alu* elements (greater than 75%, on average), which promotes efficient base pairing during crossover. (3) The vast number of *Alu* elements that create numerous identical DNA stretches, increasing the probability for recombination. (4) A chi-like motif within the *Alu* sequence that may stimulate recombination. Since 1999, approximately 25 new *Alu-Alu* recombination events have been linked to human disease. This makes the updated contribution of *Alu-Alu* recombination (both germline and somatic) to human genetic disease 0.17% (74/44,000).

Alu elements have also been linked to the presence of gene-rich segmental duplications within the human genome [41]. Given that 5–6% of the human genome sequence was created through segmental duplication events, *Alu-Alu* recombination may have contributed significantly to altered gene expression and species evolution [41]. In addition, mobile element recombination may occur in regions devoid of genes and still impact gene expression [42]. The fact that gene expression can be altered by the recombination of non-coding DNA is especially interesting since it is estimated that over 40 polymorphic *Alu-Alu* recombination events exist within humans (unpublished data). *Alu-Alu* recombination may therefore play a significant role in determining individual- and population-specific disease susceptibility.

Novel Mechanisms of *Alu*-Mediated Genomic Instability

Two novel mechanisms of *Alu*-associated genomic instability have recently been reported, *Alu* retrotransposition-mediated deletion [22] and gene conversion-mediated deletion [43]. Both mechanisms involve the retrotransposition of a new *Alu* element coupled to the deletion of genomic material at the target integration site. *Alu* retrotransposition-mediated deletion involves the integration of an *Alu* cDNA transcript at a new site in the genome, similar to the retrotransposition-mediated deletion mechanism of L1. Gene conversion-mediated deletion involves the non-reciprocal conversion of an older *Alu* element into a younger *Alu* element. Due to the retrotransposition activity of *Alu* elements within humans over the last five million years, numerous chances have arisen for both types of deletion-inducing events.

A recent study of retrotransposition-mediated deletion determined that approximately 9,000 bases of human DNA have been deleted through this

process [22]. In one instance, a 1002-bp deletion caused the functional loss of a retroviral transforming gene, *c-rel*, within the human lineage [22]. Research indicates that *c-rel* may have important roles in regulating cell proliferation and differentiation [44]. If the entire primate order is taken into account, approximately one megabase of DNA may have been deleted through *Alu* retrotransposition-mediated deletion since *Alu* elements evolved 65 million years ago.

Gene conversion-mediated deletion events have yet to be studied in such detail, although preliminary data suggest this mechanism could be as prevalent, if not more, than retrotransposition-mediated deletion (unpublished). The first published example of exonic disruption mediated by gene-conversion deletion occurred in the *CMAH* gene in humans [45]. The deletion event encompassed a 92-bp exon encoding CMP-*N*-acetylneuraminic acid hydroxylase. The partial deletion of *CMAH* induced a biochemical difference in a sialic acid cell surface receptor between humans and non-human primates. Only two other examples of gene conversion-mediated deletion have been reported to date, and arise from the young *Alu*Yg6 and Yb8 subfamilies [43, 46]. Given the fact that *Alu* elements tend to reside in gene rich regions, gene conversion-mediated deletion by young *Alu* family members may be responsible for the deletion of other exonic or regulatory regions within the human genome.

SVA Elements

The SVA element is the least well-documented retrotransposon residing within the human genome. First reported in 1994, SVA elements are a composite retrotransposon consisting of a SINE-R element, a variable number of tandem repeats (VNTR) section and an *Alu* component, all contained within direct repeats (fig. 1c) (see [8]). A recent computational study of SVA elements indicated that there are approximately 1,750–3,500 SVA elements in the human haploid genome, substantially fewer than other retrotransposons such as *Alu* and L1. Low nucleotide sequence divergences within the SVA family suggest that their small number may be the result of their recent proliferation and origin, rather than low retrotranspositional activity. SVA retrotransposition has been verified from studies documenting their involvement in the induction of disease states. Previous research has revealed the presence of an SVA-mediated transduction within the α-spectrin gene (*SPTA1*) [8]. Two other cases of disease-causing SVA insertions have also been reported. The first describes an SVA insertion into an intron of the *BTK* gene, resulting in immunodeficiency X-linked agammaglobulinemia (XLA) [8]. The second case was reported as a cause for Fukuyama-type congenital muscular dystrophy, following disruption of the fukutin gene (see review [8]).

Collectively, L1, *Alu* and SVA retrotransposable elements are responsible for 0.27% (118/44,000) of all human mutations discovered to date. They introduce genetic variation, and disease, on occasion, to human beings via an array of interesting mechanisms. Although researchers in the field of human genetics have explored the major mutational mechanisms of retrotransposable elements, their overall contribution to genomic diversity remains to be quantified.

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References

1 International Human Genome Sequencing Consortium: Initial sequencing and analysis of the human genome. Nature 2001;409:860–921.
2 Wessler SR: Plant transposable elements. A hard act to follow. Plant Physiol 2001;125:149–151.
3 Kaminker JS, Bergman CM, Kronmiller B, Carlson J, Svirskas R, Patel S, Frise E, Wheeler DA, Lewis SE, Rubin GM, Ashburner M, Celniker SE: The transposable elements of the *Drosophila melanogaster* euchromatin: a genomics perspective. Genome Biol 2002;3:RESEARCH0084.
4 Kleckner N: Transposable elements in prokaryotes. Annu Rev Genet 1981;15:341–404.
5 Batzer MA, Deininger PL: Alu repeats and human genomic diversity. Nat Rev Genet 2002;3: 370–379.
6 Ostertag EM, Kazazian HH Jr: Biology of mammalian L1 retrotransposons. Annu Rev Genet 2001;35:501–538.
7 Boeke JD: LINEs and Alus – the polyA connection. Nat Genet 1997;16:6–7.
8 Ostertag EM, Goodier JL, Zhang Y, Kazazian HH Jr: SVA elements are nonautonomous retrotransposons that cause disease in humans. Am J Hum Genet 2003;73:1444–1451.
9 Orgel LE, Crick FH: Selfish DNA: the ultimate parasite. Nature 1980;284:604–607.
10 Jelinek WR, Toomey TP, Leinwand L, Duncan CH, Biro PA, Choudary PV, Weissman SM, Rubin CM, Houck CM, Deininger PL, Schmid CW: Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc Natl Acad Sci USA 1980;77:1398–1402.
11 Morrish TA, Gilbert N, Myers JS, Vincent BJ, Stamato TD, Taccioli GE, Batzer MA, Moran JV: DNA repair mediated by endonuclease-independent LINE-1 retrotransposition. Nat Genet 2002;31:159–165.
12 Chu WM, Ballard R, Carpick BW, Williams BR, Schmid CW: Potential Alu function: regulation of the activity of double-stranded RNA-activated kinase PKR. Mol Cell Biol 1998;18:58–68.
13 Myers JS, Vincent BJ, Udall H, Watkins WS, Morrish TA, Kilroy GE, Swergold GD, Henke J, Henke L, Moran JV, Jorde LB, Batzer MA: A comprehensive analysis of recently integrated human Ta L1 elements. Am J Hum Genet 2002;71:312–326.
14 Martinez-Garay I, Ballesta MJ, Oltra S, Orellana C, Palomeque A, Molto MD, Prieto F, Martinez F: Intronic L1 insertion and F268S, novel mutations in *RPS6KA3* (RSK2) causing Coffin-Lowry syndrome. Clin Genet 2003;64:491–496.
15 van den Hurk JA, van de Pol DJ, Wissinger B, van Driel MA, Hoefsloot LH, de Wijs IJ, van den Born LI, Heckenlively JR, Brunner HG, Zrenner E, Ropers HH, Cremers FP: Novel types of mutation in the choroideremia (*CHM*) gene: a full-length L1 insertion and an intronic mutation activating a cryptic exon. Hum Genet 2003;113:268–275.

16 Mukherjee S, Mukhopadhyay A, Banerjee D, Chandak GR, Ray K: Molecular pathology of haemophilia B: identification of five novel mutations including a LINE 1 insertion in Indian patients. Haemophilia 2004;10:259–263.
17 Han JS, Szak ST, Boeke JD: Transcriptional disruption by the L1 retrotransposon and implications for mammalian transcriptomes. Nature 2004;429:268–274.
18 Perepelitsa-Belancio V, Deininger P: RNA truncation by premature polyadenylation attenuates human mobile element activity. Nat Genet 2003;35:363–366.
19 Kazazian HH Jr, Goodier JL: LINE drive. Retrotransposition and genome instability. Cell 2002;110:277–280.
20 Gilbert N, Lutz-Prigge S, Moran JV: Genomic deletions created upon LINE-1 retrotransposition. Cell 2002;110:315–325.
21 Symer DE, Connelly C, Szak ST, Caputo EM, Cost GJ, Parmigiani G, Boeke JD: Human L1 retrotransposition is associated with genetic instability in vivo. Cell 2002;110:327–338.
22 Callinan PA, Jianxin W, Herke SW, Garber RK, Liang P, Batzer MA: *Alu* retrotransposition-mediated deletion. J Mol Biol 2005;348:791–800.
23 Narita N, Nishio H, Kitoh Y, Ishikawa Y, Minami R, Nakamura H, Matsuo M: Insertion of a 5′ truncated L1 element into the 3′ end of exon 44 of the dystrophin gene resulted in skipping of the exon during splicing in a case of Duchenne muscular dystrophy. J Clin Invest 1993;91:1862–1867.
24 Kondo-Iida E, Kobayashi K, Watanabe M, Sasaki J, Kumagai T, Koide H, Saito K, Osawa M, Nakamura Y, Toda T: Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (*FCMD*). Hum Mol Genet 1999;8:2303–2309.
25 Ho HJ, Ray DA, Salem AH, Myers JS, Batzer MA: Straightening out the LINEs: LINE-1 orthologous loci. Genomics 2005;85:201–207.
26 Vincent BJ, Myers JS, Ho HJ, Kilroy GE, Walker JA, Watkins WS, Jorde LB, Batzer MA: Following the LINEs: an analysis of primate genomic variation at human-specific LINE-1 insertion sites. Mol Biol Evol 2003;20:1338–1348.
27 Holmes SE, Dombroski BA, Krebs CM, Boehm CD, Kazazian HH Jr: A new retrotransposable human L1 element from the LRE2 locus on chromosome 1q produces a chimaeric insertion. Nat Genet 1994;7:143–148.
28 Moran JV, DeBerardinis RJ, Kazazian HH Jr: Exon shuffling by L1 retrotransposition. Science 1999;283:1530–1534.
29 Goodier JL, Ostertag EM, Kazazian HH Jr: Transduction of 3′-flanking sequences is common in L1 retrotransposition. Hum Mol Genet 2000;9:653–657.
30 Deininger PL, Batzer MA: Evolution of retroposons; in Hecht MK (ed.): Evolutionary Biology. Plenum Press, New York, 1993, pp 157–196.
31 Mitchell GA, Labuda D, Fontaine G, Saudubray JM, Bonnefont JP, Lyonnet S, Brody LC, Steel G, Obie C, Valle D: Splice-mediated insertion of an Alu sequence inactivates ornithine delta-aminotransferase: a role for *Alu* elements in human mutation. Proc Natl Acad Sci USA 1991;88:815–819.
32 Lev-Maor G, Sorek R, Shomron N, Ast G: The birth of an alternatively spliced exon: 3′ splice-site selection in *Alu* exons. Science 2003;300:1288–1291.
33 Ganguly A, Dunbar T, Chen P, Godmilow L, Ganguly T: Exon skipping caused by an intronic insertion of a young *Alu* Yb9 element leads to severe hemophilia A. Hum Genet 2003;113:348–352.
34 Knebelmann B, Forestier L, Drouot L, Quinones S, Chuet C, Benessy F, Saus J, Antignac C: Splice-mediated insertion of an *Alu* sequence in the *COL4A3* mRNA causing autosomal recessive Alport syndrome. Hum Mol Genet 1995;4:675–679.
35 Ferlini A, Galie N, Merlini L, Sewry C, Branzi A, Muntoni F: A novel *Alu*-like element rearranged in the dystrophin gene causes a splicing mutation in a family with X-linked dilated cardiomyopathy. Am J Hum Genet 1998;63:436–446.
36 Vervoort R, Gitzelmann R, Lissens W, Liebaers I: A mutation (IVS8+0.6 kbdelTC) creating a new donor splice site activates a cryptic exon in an *Alu*-element in intron 8 of the human beta-glucuronidase gene. Hum Genet 1998;103:686–693.
37 Kreahling J, Graveley BR: The origins and implications of *Alu*ternative splicing. Trends Genet 2004;20:1–4.

38 Claverie-Martin F, Gonzalez-Acosta H, Flores C, Anton-Gamero M, Garcia-Nieto V: De novo insertion of an *Alu* sequence in the coding region of the *CLCN5* gene results in Dent's disease. Hum Genet 2003;113:480–485.
39 Sukarova E, Dimovski AJ, Tchacarova P, Petkov GH, Efremov GD: An *Alu* insert as the cause of a severe form of hemophilia A. Acta Haematol 2001;106:126–129.
40 Deininger PL, Batzer MA: *Alu* repeats and human disease. Mol Genet Metab 1999;67:183–193.
41 Bailey JA, Liu G, Eichler EE: An *Alu* transposition model for the origin and expansion of human segmental duplications. Am J Hum Genet 2003;73:823–834.
42 Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, Dioszegi M, Dikkers FG, Hildering P, Willems PJ, Verheij JB, Lindpaintner K, Vickery B, Foernzler D, Van Hul W: Identification of a 52 kb deletion downstream of the *SOST* gene in patients with van Buchem disease. J Med Genet 2002;39:91–97.
43 Salem AH, Kilroy GE, Watkins WS, Jorde LB, Batzer MA: Recently integrated *Alu* elements and human genomic diversity. Mol Biol Evol 2003;20:1349–1361.
44 Bishop JM, Varmus H: Functions and origins of retroviral oncogenes; in Weiss R, Teich N, Varmus H, Coffin J (eds): Molecular Biology of the Tumor Viruses: RNA Tumor Viruses. Cold Spring Harbor, New York, 1982, pp 999–1108.
45 Hayakawa T, Satta Y, Gagneux P, Varki A, Takahata N: Alu-mediation inactivation of the human CMP-N-acetyl-neuraminic acid hydroxylase gene. Proc Natl Acad Sci USA 2001;98:11399–11404.
46 Carter AB, Salem A-H, Hedges DJ, Nguyen Keegan C, Kimball B, Walker JA, Watkins WS, Jorde LB, Batzer MA: Genome wide analysis of the human Yb lineage. Hum Genomics 2004;1:167–168.

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The Spindle Checkpoint and Chromosomal Stability

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Abstract

Normal human somatic cells contain 46 chromosomes (22 pairs of autosomes and two sex chromosomes). Chromosome missegregation leads to abnormal numbers of chromosomes or aneuploidy. This form of genetic instability alters the dosages of large subsets of genes, which can result in severe disease phenotypes. Most human cancer cells are aneuploid. It is generally believed that aneuploidy contributes to cancer formation. The spindle checkpoint is a cell-cycle surveillance mechanism that ensures the fidelity of chromosome segregation during mitosis and meiosis. In this article, we review our current understanding of the molecular basis of the spindle checkpoint and the recent evidence that links the malfunction of this checkpoint to aneuploidy and tumorigenesis.

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Cancer has long been recognized as a disease associated with genetic instability. A prevalent form of genetic instability in human cancers is chromosome instability (CIN) [1, 2]. As compared to normal cells, cancer cells with CIN gain or lose their chromosomes at a higher rate and contain abnormal numbers of chromosomes (aneuploidy). The molecular basis of CIN is not yet fully understood. However, it has become increasingly clear that malfunction of a cell-cycle surveillance mechanism called the spindle checkpoint contributes to CIN and aneuploidy.

The Spindle Checkpoint

The chromosomes are duplicated once and only once during each cell division [3]. The duplicated chromosomes are physically tethered together by the

cohesin protein complex and are packaged into sister chromatids during mitosis [3]. In mitosis of animal cells, microtubules emanating from the two spindle poles are dynamically growing and shrinking [4]. The microtubules that are captured by the kinetochores (protein complexes that are assembled at the centromeres) of sister chromatids become selectively stabilized and link the chromosome to that pole [4]. Once the two opposing kinetochores of a given pair of sister chromatids are captured by microtubules from the two opposite spindle poles, that pair of sister chromatids becomes bi-orientated and aligned at the equator of the mitotic spindle [3, 4]. After all pairs of sister chromatids achieve bi-orientation and their kinetochores are under tension as a result of their attachment to the bipolar spindle, an E3 ubiquitin ligase complex called the anaphase-promoting complex or cyclosome (APC/C) tags the securin protein with polyubiquitin chains and targets it for degradation by the proteasome (fig. 1) [3, 5]. Securin is an inhibitory chaperone of a cysteine protease called separase, because securin facilitates the folding of separase and also inhibits its protease activity [5]. Degradation of securin leads to the activation of separase which then cleaves the Scc1 subunit of cohesin [5]. This resolves the linkage between sister chromatids (fig. 1). The separated sister chromatids are then pulled to opposite spindle poles through their attachment to spindle microtubule fibers. This elegant process ensures that the two daughter cells inherit identical sets of chromosomes [3, 5].

On the other hand, the stochastic 'search-and-capture' mechanism of kinetochore-microtubule attachment implies that not all sister chromatids can achieve bi-orientation synchronously [4]. Thus, cells employ a surveillance mechanism called the spindle checkpoint to prevent premature sister chromatid separation prior to the bi-orientation of all pairs of sister chromatids. Because APC/C-mediated degradation of securin is the first irreversible step in initiating sister chromatid separation, it is not surprising that APC/C is the critical molecular target of the spindle checkpoint [6, 7]. Kinetochores that are not attached by microtubules and not under tension emit a diffusible signal that inhibits the cytoplasmic pool of APC/C, therefore preventing premature chromosome segregation. Thus, the spindle checkpoint is active in each and every cell cycle, and is important for cells to maintain the accuracy and fidelity of chromosome segregation [6–8].

The activity of APC/C is controlled by two related activators: Cdc20 and Cdh1. Both Cdc20 and Cdh1 contain a C-terminal WD40-repeat domain, and are involved in recruiting substrates to APC/C [5]. APC/C^{Cdc20} and APC/C^{Cdh1} perform distinct functions and are differentially regulated during the cell cycle [5]. APC/C^{Cdc20} is required for sister chromatid separation and the metaphase–anaphase transition by ubiquitinating securin whereas APC/C^{Cdh1} mediates the degradation of a broader spectrum of substrates in late anaphase and early G1 [5]. The spindle checkpoint selectively inhibits the activity of

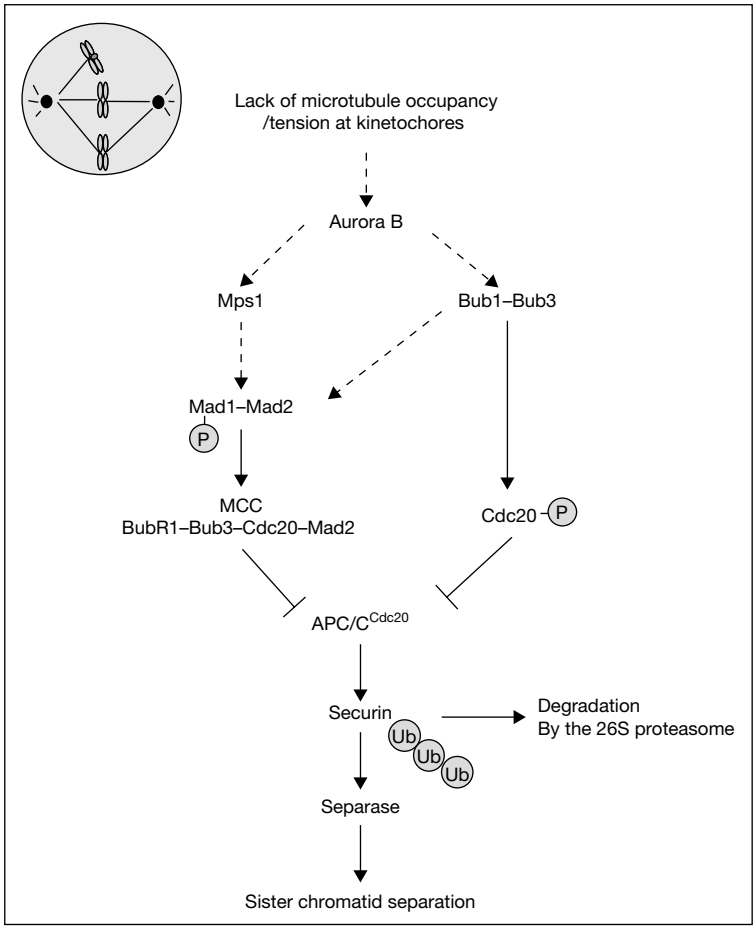


Fig. 1. Molecular pathways of the spindle checkpoint. At the metaphase–anaphase transition, APC/C^{Cdc20} ubiquitinates securin. Degradation of securin activates separase. Separase then cleaves the Scc1 subunit of cohesin, allowing chromosome segregation. In response to sister chromatid not properly attached to the mitotic spindle, the spindle checkpoint promotes the assembly of MCC that inhibits the activity of APC/C in a stoichiometric manner. The Bub1 spindle checkpoint kinase also becomes active, phosphorylates Cdc20, and inhibits the activity of APC/C catalytically. Inhibition of APC/C stabilizes securin, preserves sister chromatid cohesion, and delays the onset of anaphase until all sister chromatids have achieved bi-orientation on the mitotic spindle.

APC/C^{Cdc20}, stabilizes securin, delays the activation of separase, and prevents premature sister chromatid separation [6–8].

Molecular Mechanism of the Spindle Checkpoint

Components of the Spindle Checkpoint

The molecular players of the spindle checkpoint include Mitotic Arrest Deficiency 1 (Mad1), Mad2, Mad3/BubR1, Budding Uninhibited by Benzimidazole 1 (Bub1), Bub3, and Monopolar Spindle 1 (Mps1). The Bub and Mad genes were identified in two separate yeast genetic screens [9, 10]. Yeast cells that harbor mutations in these genes fail to arrest in mitosis upon transient exposure to microtubule destabilizing drugs, such as nocodazole and benzimidazole, and therefore lose viability. Bub3 was found as a suppressor of the *bub1-1* allele in a suppressor screen [9]. *MPS1* was originally identified as a gene required for spindle pole body duplication [11]. The *mps1* mutant cells contain monopolar spindle, but do not arrest the cell cycle at metaphase [12]. Thus, Mps1 is also required for the spindle checkpoint.

Homologues of the yeast spindle checkpoint genes were later identified in higher organisms [6–8]. Disruption of these checkpoint genes in mammalian cells by antibody injection, antisense oligonucleotides, or RNA interference (RNAi) increases the frequency of chromosome missegregation during the normal cell cycle and causes a failure of these cells to undergo prolonged mitotic arrest in the presence of microtubule poisons, such as nocodazole and taxol [6–8]. In yeast, cells with the spindle checkpoint genes deleted are still viable, although the *bub1*- and *bub3*-null cells are very sick and have higher rates of chromosome loss [13, 14]. In contrast, these spindle checkpoint genes are essential in higher organisms. For example, flies homozygous for P element-induced, near-null mutations of *BubR1* die during late larval/pupal stages due to chromosome missegregation and apoptosis [15, 16]. The *Mad2*-null embryonic cells are unable to arrest in response to spindle disruption [17]. *Mad2* knockout mice are embryonic lethal [17]. The widespread chromosome missegregation and apoptosis occur in *Mad2*-null embryos at E6.5 when the cells of the epiblast begin rapid cell divisions [17]. *BubR1*-deficient mice also die before E8.5 due to massive chromosome missegregation and apoptosis [18]. Thus, the spindle checkpoint is required for accurate chromosome segregation in fly, mouse, and human cells in the absence of spindle damage.

In addition to these core components, several other proteins play important roles in the spindle checkpoint. For example, Aurora B is a protein kinase that is required for proper chromosome segregation and cytokinesis. The Aurora B–INCENP complex senses the lack of tension at kinetochores and destabilizes

erroneous kinetochore–microtubule attachments [19, 20]. Centromere-associated protein E (CENP-E) is a mitotic kinesin required for efficient, stable microtubule capture at kinetochores [21, 22]. CENP-E directly binds to BubR1 and activates the kinase activity of BubR1 [23]. The exact function of the CENP-E–BubR1 interaction in the spindle checkpoint is unclear at present.

Kinetochore Localization of Spindle Checkpoint Proteins

The kinetochore is the origin of the 'wait-anaphase signal' and its correct assembly is required for proper spindle checkpoint function [4]. All known spindle checkpoint proteins are dynamically associated with kinetochores in mitosis [4, 24]. The concentrations of many checkpoint proteins at unattached kinetochores are higher than those of attached ones. This correlates well with the on-and-off status of the spindle checkpoint. Moreover, the kinetochore localization of checkpoint proteins has been shown to be essential for the spindle checkpoint [4, 7]. However, few direct interactions between the spindle checkpoint proteins and core components of the kinetochore have been identified. In budding yeast, Skp1, an essential component for kinetochore assembly, was shown to interact with Bub1 directly and is responsible for recruiting Bub1 to the kinetochore [25]. Unfortunately, Skp1 proteins in other organisms have not been shown to be involved in kinetochore function. It remains to be determined whether the Skp1–Bub1 interaction is conserved in organisms other than budding yeast. The Ndc80 kinetochore complex is required for Mad1 localization at the kinetochores, but direct interactions between them have not been demonstrated [26, 27].

On the other hand, studies in *Xenopus* egg extracts have revealed the hierarchy and temporal order of binding of checkpoint proteins to kinetochores in mitosis [28–31]. Aurora B lies most upstream in this process and is required for the kinetochore localization of all other checkpoint proteins. Bub1, Mps1, and CENP-E are recruited next and their kinetochore localizations are interdependent [28, 32]. Bub1 interacts with Bub3 throughout the cell cycle and their kinetochore localization is interdependent [30]. Mad1 and Mad2 also form a complex throughout the cell cycle [31]. Mad1 is required for the kinetochore association of Mad2 [31]. Mad2, Cdc20, and APC/C are the downstream components [28]. They are recruited to kinetochores separately and their kinetochore localization is dependent on the upstream components [28]. Similar results were obtained in mammalian cells by using RNAi to knockdown certain spindle checkpoint proteins and examining the kinetochore localization of others [33, 34]. Aurora B is required for the kinetochore localization of Bub1 [34]. Bub1, BubR1, CENP-E, and Mad2 are then recruited to kinetochores sequentially [34]. Together with genetic analysis in yeast, these results indicate that Aurora B, Bub1, and Mps1 act upstream in the spindle checkpoint pathway

(fig. 1). These studies also suggest that kinetochore-dependent conformational changes or post-translational modifications of these upstream checkpoint proteins are required for the kinetochore recruitment and activation of the downstream proteins.

Inhibition of APC/C by the Mitotic Checkpoint Complex (MCC)

Among all the spindle checkpoint proteins, the biochemical function of Mad2 was revealed first. Deletion of Mad2 in yeast abolishes the spindle checkpoint [10, 35]. It was shown that Mad2 binds directly to Cdc20 in both budding and fission yeast [36, 37]. Fission yeast cells harboring a Slp1 (the Cdc20 ortholog in fission yeast) mutant that cannot bind to Mad2 escape from the mitotic arrest exerted by Mad2 overexpression [36]. These findings suggest that Cdc20 is a critical downstream target of Mad2. Subsequent biochemical studies in *Xenopus* egg extracts and mammalian cells showed that Cdc20 is an activator of the ubiquitin ligase activity of APC/C [38]. Mad2, Cdc20, and APC/C form a ternary complex upon checkpoint activation and the ubiquitin ligase activity of APC/C is inhibited in this complex [39]. Moreover, recombinant purified Mad2 protein exists in both monomeric and dimeric forms. Mad2 dimer, but not Mad2 monomer, inhibits APC/C^{Cdc20} in *Xenopus* egg extracts, suggesting that either dimerization and/or a conformational change of Mad2 are required for APC/C inhibition [39].

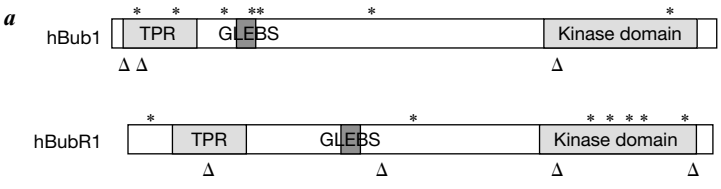
The structures of apo-Mad2 and Mad2 in complex with a peptide that mimics the Mad2-binding motifs of Mad1 and Cdc20 were then determined by nuclear magnetic resonance (NMR) spectroscopy [33, 40]. These studies revealed a dramatic conformational change between apo-Mad2 and Mad2 bound to Mad1 or Cdc20. The crystal structure of Mad2 bound to a 120-residue fragment of Mad1 was then determined and confirmed that Mad1 and Cdc20 trigger similar conformational changes of Mad2 [41]. Recently, Luo et al. have shown that the monomeric and dimeric forms of Mad2 interconvert slowly in vitro in the absence of ligands [42]. This interconvertion is accelerated by a sub-stoichiometric amount of Mad2-binding peptide of Mad1. The dimeric form of Mad2 has a conformation similar to the Cdc20-bound form of Mad2 and is thus more active in inhibiting APC/C^{Cdc20} [42]. These and other studies led to a 'two-state Mad2' model. In this model, Mad2 exists in two conformations, one of which is more active in inhibiting APC/C^{Cdc20}. Upon checkpoint activation, Mad1 facilitates the formation of the 'activated' conformation of Mad2, leading to inhibition of APC/C. Very recently, a related but distinct model called the 'Mad1–Mad2 template' model has been proposed [43]. In this model, the Mad1–Mad2 heterodimer recruits another Mad2 molecule through a Mad2–Mad2 interaction. The loosely bound Mad2 molecule is passed on to Cdc20, resulting in APC/C inhibition. Obviously, more studies are needed to test both models.

BubR1, the vertebrate homolog of yeast Mad3, was next shown to be another direct inhibitor of APC/C [44]. Similar to Mad3, BubR1 associates with Bub3 constitutively via its conserved GLEBS motif [45]. Unlike Mad3 that does not have any catalytic domain, BubR1 has a C-terminal kinase domain. Purified recombinant BubR1 protein inhibited the activity of APC/C immunoprecipitated from *Xenopus* egg extracts and mammalian cells in the absence of Mad2 [44]. Surprisingly, direct binding between BubR1 and Cdc20, but not the kinase activity of BubR1, is responsible for the APC/C-inhibitory effect of BubR1 [44]. Although both Mad2 and BubR1 can inhibit Cdc20 independently, there is synergism between them in inhibiting APC/C in vitro [46]. Moreover, BubR1/Mad3, Mad2, Bub3, and Cdc20 form a single complex called the mitotic checkpoint complex (MCC) in mitosis [29, 47, 48]. Thus, the MCC is a key stoichiometric checkpoint inhibitor of APC/C in vivo (fig. 1). On the other hand, the fact that the BubR1–Bub3–Cdc20 and Mad2–Cdc20 sub-complexes are sufficient to inhibit APC/C in vitro suggests that these sub-complexes might also be involved in APC/C inhibition in response to different spindle defects in vivo.

The Multiple Functions of Bub1 in the Spindle Checkpoint

Bub1 is a protein kinase that plays multiple roles in the spindle checkpoint. Bub1 contains three major domains: an N-terminal tetratrico peptide repeat (TPR) domain that mediates its kinetochore localization, a GLEBS (GLE2p-binding sequence) motif that binds to Bub3, and a C-terminal serine/threonine kinase domain (fig. 2a). Studies in budding yeast have firmly established the requirement of Bub1 for proper spindle checkpoint function [9]. A dominant allele of Bub1 that harbors a point mutation in its kinase domain exhibits higher kinase activity and, when overexpressed in cells, causes a Mad2-dependent mitotic delay [49]. Furthermore, Mad1 becomes hyperphosphorylated in response to spindle checkpoint activation in budding yeast [50]. Bub1–Bub3 forms a complex with Mad1 upon checkpoint activation in yeast [51]. Human Bub1 phosphorylates Mad1 in vitro, although its in vivo relevance and functions have not been established [52]. As discussed above, Bub1 is also required for the kinetochore localization of Mad1 and Mad2 in vertebrates. These results strongly suggest that Bub1 acts upstream of Mad1 and Mad2 (fig. 1).

A recent study by Tang et al. modified this classical view of Bub1 and revealed a downstream function of Bub1 [53]. Tang et al. showed that Bub1 inhibits the APC/C^{Cdc20} activity in an ATP-dependent fashion by directly phosphorylating Cdc20 in vitro. Furthermore, Cdc20 is phosphorylated at six sites in vivo. Recombinant Bub1 phosphorylates Cdc20 at the same six sites in vitro. A Cdc20 mutant that lacks these phosphorylation sites (referred to as $Cdc20^{BPM}$) is refractory to phosphorylation and inhibition by Bub1 in vitro. When expressed in cells, $Cdc20^{BPM}$ also abrogates the ability of these cells to undergo mitotic



b

Protein	Mutation	Source tissues or cell lines	Reference
Bub1	Δ10-32 a.a.	Lymphoid leukemia cell line Molt3	[69]
	H51D	Lung adenocarcinoma	[68]
	Δ76-1085 a.a.	Colorectal cancer cell line V400	[59]
	A130S	Colorectal cancer	[76]
	R209Q	Lung cancer cell line NCI-H345	[71]
	Y259C, H265N	Pancreatic cancer cell line Hs766T	[70]
	S492Y	Colorectal cancer cell line V429	[59]
	Δ827-1085 a.a.	Thyroid follicular lymphoma	[67]
	S950G	Rectal cancer	[72]
BubR1	T40M	Colorectal cancer cell line V531	[59]
	ΔT1023	Colorectal cancer cell line V1394	[59]
	Δ194-1050 a.a.	MVA	[81]
	Δ483-1050 a.a.	MVA	[81]
	R550Q	MVA	[81]
	Δ738-1050 a.a.	MVA	[81]
	R814H	MVA	[81]
	L844F, Q921H	MVA	[81]
	I909T	MVA	[81]
	L1012P	MVA	[81]

Fig. 2. Domain structure and mutations of human Bub1 and BubR1. ***a*** Bub1 and BubR1 contain an N-terminal tetratricopeptide repeat (TPR) motif, a GLEBS motif for association with Bub3, and a C-terminal kinase domain. Missense and nonsense mutations found in human cancers are indicated by asterisks and triangles, respectively. ***b*** List of Bub1 and BubR1 mutations found in human tumors and MVA patients.

arrest in the presence of spindle poisons [53]. Moreover, $Cdc20^{BPM}$ is still capable of interacting with and being inhibited by BubR1 or Mad2, although it remains possible that Bub1-mediated phosphorylation of Cdc20 might facilitate the formation of MCC in vivo [53]. These results indicate that phosphorylation of Cdc20 by Bub1 is required for efficient checkpoint signaling (fig. 1).

Consistent with an involvement of the Bub1 kinase activity in the spindle checkpoint, Bub1 itself is rapidly phosphorylated upon spindle damage caused by brief treatments with nocodazole or taxol [54]. The kinase activity of Bub1

toward Cdc20 is enhanced in mitosis [53]. Furthermore, Bub1 becomes hyperphosphorylated when bound to chromatin, and the kinase activity of chromosome-bound Bub1 is enhanced [55]. The mechanism by which Bub1 is activated upon checkpoint activation is unknown. Bub1 has been shown to be phosphorylated by Cdk1 in fission yeast [56]. However, it is unclear whether Cdk1-mediated phosphorylation enhances the kinase activity of Bub1.

In summary, the spindle checkpoint uses multiple mechanisms to inhibit APC/C. MCC (BubR1–Bub3–Cdc20–Mad2) or its sub-complexes inhibit APC/C in a stoichiometric manner whereas Bub1 inhibits APC/C catalytically. Consistent with this notion, fluorescence recovery after photobleaching (FRAP) experiments in live cells show that GFP-Cdc20 displays a biphasic dynamics at unattached kinetochores: a slow phase that is likely to reflect the formation and release of MCC and a fast phase that might be due to the release of free Cdc20 [57]. It is conceivable that the more dynamic population of Cdc20 is phosphorylated and inhibited by Bub1 and released before it can be incorporated into MCC. This catalytic mechanism for APC/C^{Cdc20} inhibition may help to explain how only one unattached kinetochore can generate enough 'wait-anaphase signal' to inhibit the entire pool of APC/C^{Cdc20} in the cell.

Defective Spindle Checkpoint and Aneuploidy

Mutations of Spindle Checkpoint Genes in Cancer

A growing body of evidence indicates that defects in spindle checkpoint signaling might contribute to tumorigenesis. It is well accepted that cancer formation requires multiple genetic alterations [2]. Cells harboring mutations that allow them to gain growth advantage will expand in the population. The number of mutations that are necessary for clonal expansion and cancer formation is estimated by mathematical extrapolation to be between 6 and 10 [2]. However, the DNA mutation rate in normal cells is too low for this number of mutations to occur in the lifetime of a given cell. If the spindle checkpoint is compromised, cells may gain or lose chromosomes in each and every division. This may lead to the loss-of-heterozygosity (LOH) of tumor suppressors and/or the acquisition of extra copies of proto-oncogenes, thus accelerating the transformation process. Thus, the spindle checkpoint is proposed to be a barrier that cells need to overcome to become cancerous [58].

Indeed, mutations in *BUB1* might be involved in the pathogenesis of human cancers, especially colorectal cancer. Mutations in one of the two copies of *BUB1* were found in CIN-type colon cancer cells [59] (fig. 2). Cahill et al. described two *BUB1* mutations: one encodes a truncated Bub1 protein containing only the N-terminal 75 residues while the other introduces a single point

mutation at residue 492 that changes the serine to tyrosine [59]. Interestingly, expression of the mutated Bub1 protein containing only residues 1–75 disrupted the spindle checkpoint in euploid colon cancer cells and caused aneuploidy, presumably through blocking the functions of the wild-type Bub1 in a dominant-negative fashion [59]. Though the exact mechanism of this dominant-negative effect of Bub1 is unknown, it is conceivable that the activation of Bub1 involves autophosphorylation through a dimerization event at the kinetochores, akin to the activation of receptor tyrosine kinases [60]. The N-terminal 75-residue fragment of Bub1 might bind to the wild-type Bub1 and prevent its activation and/or kinetochore localization.

Subsequently, mutations of *BUB1* were extensively studied in different cancer samples and cell lines with aneuploidy. However, mutations of *BUB1* were found to be rare in glioblastomas [61], bladder tumors, soft-tissue sarcomas, hepatocellular carcinomas [62], head and neck squamous cell carcinomas [63], gastric carcinomas [64], and breast carcinomas [65]. On the other hand, the *BUB1* genomic locus on 2q14 was shown to be relatively unstable in 14.5% of colorectal cancer samples [66]. The Bub1 mutations identified so far in tumor samples and cell lines are mainly located in its N-terminal kinetochore localization domain [67–70] (fig. 2). However, it has not been clearly demonstrated whether these mutations affect the kinetochore localization of Bub1. Mutations in *BUBR1*/*BUB1B* and *MAD2* were also examined in cancer cell lines and tumor samples. Similar to *BUB1*, *BUBR1* and *MAD2* are not frequently mutated in multiple cancers [62, 71, 72].

Involvement of the spindle checkpoint genes in cancer formation was also confirmed in mouse models. As mentioned above, *Mad2*-null mice are not viable. However, *Mad2*$^{+/-}$ heterozygous mice are viable and develop lung cancers after a long latency period [73]. Similarly, *BubR1*-null mutations also cause lethality in mice. The *BubR1*$^{+/-}$ mice also develop aneuploidy and form lung and intestinal adenocarcinomas when they are challenged with carcinogens [74]. Finally, *Bub1* mutations foster growth and cellular transformation of cells derived from *Brca2*-deficient mice. Tumors from *Brca2*-deficient mice showed spindle checkpoint dysfunction and contained mutations in *Bub1* and *BubR1* [75]. These results suggest that defects in the spindle checkpoint are involved in the initiation and/or progression of cancers.

Epigenetic Changes

Epigenetic changes instead of genomic mutations might also lead to a defective spindle checkpoint in cancer cells and contribute to tumor progression. Alterations of expression of the spindle checkpoint genes were examined in tumor samples and cancer cell lines. A reduction in the Bub1 protein level was observed in surgically dissected colorectal carcinomas, which positively

correlated with metastasis and shorter relapse-free survival [76]. The expression levels of Bub1 and Mad2 were also changed in certain breast cancer cell lines [77, 78]. Consistently, *Mad2* is a direct transcriptional target of the BRCA1 tumor suppressor protein [79]. BRCA1 binds to the promoter of *Mad2* and up-regulates its transcription. Overexpression of *Mad2* in *Brca1*-deficient cells partially rescues the spindle checkpoint defects [79]. Intriguingly, *MAD2* is also a transcriptional target of E2F [80]. The function of E2F is often up-regulated in cancers either through its own overexpression or through the loss of its inhibitor, pRb. Consequently, *MAD2* is constitutively expressed and deregulated in *RB*-deficient cells and these cells exhibited accelerated mitosis and chromosome instability [80]. Therefore, either up- or down-regulation of Mad2 levels can lead to chromosome instability and aneuploidy. It is thus crucial to maintain the correct steady state levels of Mad2 and other checkpoint proteins in cells.

Germline Mutation of BUBR1 and Cancer Predisposition

Although the spindle checkpoint genes are mutated in human cancers, it is unclear whether these somatic cell mutations are causal events that lead to cancer or simply secondary consequences of cancer progression. Very recently, studies on a rare human disease called mosaic variegated aneuploidy syndrome (MVA) provided the first direct evidence for a causal role of a defective spindle checkpoint in human cancers [81]. MVA is an autosomal recessive condition and $>25\%$ cells in multiple tissues of these patients are aneuploid. Some MVA patients have intrauterine growth retardation and microcephaly. Importantly, many MVA patients develop cancer at a young age. Hanks et al. sequenced the *BUBR1* gene in eight MVA pedigrees and found biallelic *BUBR1* mutations in five families [81]. In each family, there is a missense mutation and a mutation that results in a truncated protein or no transcripts. The five missense mutations all affect conserved residues in the kinase domain of BubR1 [81]. Because a complete loss of BubR1 function is expected to cause lethality, these missense mutations are not likely to severely disrupt the biochemical function of the remaining copy of *BUBR1*. Two of the five patients developed embryonic rhabdomyosarcomas in different tissues at the age of 7 years and 5 months, respectively [81]. These results provide the strongest evidence so far that a defective spindle checkpoint results in cancer predisposition in humans.

Conclusion

The spindle checkpoint is a cell-cycle surveillance mechanism that ensures the fidelity of chromosome segregation in mitosis and meiosis. In response to misaligned sister chromatids, the checkpoint uses multiple ways to block the

activity of APC/C, thus delaying the onset of sister chromatid separation. Malfunction of the spindle checkpoint leads to aneuploidy and contributes to cancer formation. A better understanding of the spindle checkpoint at the molecular level will be valuable for identifying new drug targets for treating human cancers.

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References

1 Lengauer C, Kinzler KW, Vogelstein B: Genetic instabilities in human cancers. Nature 1998;396: 643–649.
2 Rajagopalan H, Lengauer C: Aneuploidy and cancer. Nature 2004;432:338–341.
3 Nasmyth K: Segregating sister genomes: the molecular biology of chromosome separation. Science 2002;297:559–565.
4 Cleveland DW, Mao Y, Sullivan KF: Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell 2003;112:407–421.
5 Peters JM: The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol Cell 2002;9:931–943.
6 Yu H: Regulation of APC-Cdc20 by the spindle checkpoint. Curr Opin Cell Biol 2002;14:706–714.
7 Bharadwaj R, Yu H: The spindle checkpoint, aneuploidy, and cancer. Oncogene 2004;23:2016–2027.
8 Musacchio A, Hardwick KG: The spindle checkpoint: structural insights into dynamic signalling. Nat Rev Mol Cell Biol 2002;3:731–741.
9 Hoyt MA, Totis L, Roberts BT: *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell 1991;66:507–517.
10 Li R, Murray AW: Feedback control of mitosis in budding yeast. Cell 1991;66:519–531.
11 Winey M, Goetsch L, Baum P, Byers B: *MPS1* and *MPS2*: novel yeast genes defining distinct steps of spindle pole body duplication. J Cell Biol 1991;114:745–754.
12 Hardwick KG, Weiss E, Luca FC, Winey M, Murray AW: Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. Science 1996;273:953–956.
13 Vanoosthuyse V, Valsdottir R, Javerzat JP, Hardwick KG: Kinetochore targeting of fission yeast Mad and Bub proteins is essential for spindle checkpoint function but not for all chromosome segregation roles of Bub1p. Mol Cell Biol 2004;24:9786–9801.
14 Warren CD, Brady DM, Johnston RC, Hanna JS, Hardwick KG, Spencer FA: Distinct chromosome segregation roles for spindle checkpoint proteins. Mol Biol Cell 2002;13:3029–3041.
15 Basu J, Bousbaa H, Logarinho E, Li Z, Williams BC, Lopes C, Sunkel CE, Goldberg ML: Mutations in the essential spindle checkpoint gene *bub1* cause chromosome missegregation and fail to block apoptosis in *Drosophila*. J Cell Biol 1999;146:13–28.
16 Logarinho E, Bousbaa H, Dias JM, Lopes C, Amorim I, Antunes-Martins A, Sunkel CE: Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in *Drosophila* cells. J Cell Sci 2004;117:1757–1771.
17 Dobles M, Liberal V, Scott ML, Benezra R, Sorger PK: Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. Cell 2000;101:635–645.

18 Wang Q, Liu T, Fang Y, Xie S, Huang X, Mahmood R, et al: *BUBR1* deficiency results in abnormal megakaryopoiesis. Blood 2004;103:1278–1285.
19 Tanaka TU: Bi-orienting chromosomes on the mitotic spindle. Curr Opin Cell Biol 2002;14: 365–371.
20 Tanaka TU, Rachidi N, Janke C, Pereira G, Galova M, Schiebel E, Stark MJ, Nasmyth K: Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. Cell 2002;108:317–329.
21 Yao X, Abrieu A, Zheng Y, Sullivan KF, Cleveland DW: CENP-E forms a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint. Nat Cell Biol 2000;2: 484–491.
22 Abrieu A, Kahana JA, Wood KW, Cleveland DW: CENP-E as an essential component of the mitotic checkpoint in vitro. Cell 2000;102:817–826.
23 Mao Y, Abrieu A, Cleveland DW: Activating and silencing the mitotic checkpoint through CENP-E-dependent activation/inactivation of BubR1. Cell 2003;114:87–98.
24 Taylor SS, Scott MI, Holland AJ: The spindle checkpoint: a quality control mechanism which ensures accurate chromosome segregation. Chromosome Res 2004;12:599–616.
25 Kitagawa K, Abdulle R, Bansal PK, Cagney G, Fields S, Hieter P: Requirement of Skp1-Bub1 interaction for kinetochore-mediated activation of the spindle checkpoint. Mol Cell 2003;11: 1201–1213.
26 Martin-Lluesma S, Stucke VM, Nigg EA: Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. Science 2002;297:2267–2270.
27 Bharadwaj R, Qi W, Yu H: Identification of two novel components of the human NDC80 kinetochore complex. J Biol Chem 2004;279:13076–13085.
28 Vigneron S, Prieto S, Bernis C, Labbe JC, Castro A, Lorca T: Kinetochore localization of spindle checkpoint proteins: who controls whom? Mol Biol Cell 2004;15:4584–4596.
29 Chen RH: BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. J Cell Biol 2002;158:487–496.
30 Sharp-Baker H, Chen RH: Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. J Cell Biol 2001;153:1239–1250.
31 Chen RH, Shevchenko A, Mann M, Murray AW: Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. J Cell Biol 1998;143:283–295.
32 Abrieu A, Magnaghi-Jaulin L, Kahana JA, Peter M, Castro A, Vigneron S, Lorca T, Cleveland DW, Labbe JC: Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. Cell 2001;106:83–93.
33 Luo X, Tang Z, Rizo J, Yu H: The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. Mol Cell 2002;9:59–71.
34 Johnson VL, Scott MI, Holt SV, Hussein D, Taylor SS: Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. J Cell Sci 2004;117: 1577–1589.
35 Chen RH, Brady DM, Smith D, Murray AW, Hardwick KG: The spindle checkpoint of budding yeast depends on a tight complex between the Mad1 and Mad2 proteins. Mol Biol Cell 1999;10: 2607–2618.
36 Kim SH, Lin DP, Matsumoto S, Kitazono A, Matsumoto T: Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint. Science 1998;279:1045–1047.
37 Hwang LH, Lau LF, Smith DL, Mistrot CA, Hardwick KG, Hwang ES, Amon A, Murray AW: Budding yeast Cdc20: a target of the spindle checkpoint. Science 1998;279:1041–1044.
38 Fang G, Yu H, Kirschner MW: Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol Cell 1998;2:163–171.
39 Fang G, Yu H, Kirschner MW: The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev 1998;12:1871–1883.
40 Luo X, Fang G, Coldiron M, Lin Y, Yu H, Kirschner MW, Wagner G: Structure of the Mad2 spindle assembly checkpoint protein and its interaction with Cdc20. Nat Struct Biol 2000;7: 224–229.

41 Sironi L, Mapelli M, Knapp S, De Antoni A, Jeang KT, Musacchio A: Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint. EMBO J 2002;21:2496–2506.
42 Luo X, Tang Z, Xia G, Wassmann K, Matsumoto T, Rizo J, Yu H: The Mad2 spindle checkpoint protein has two distinct natively folded states. Nat Struct Mol Biol 2004;11:338–345.
43 De Antoni A, Pearson CG, Cimini D, Canman JC, Sala V, Nezi L, et al: The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. Curr Biol 2005;15:214–225.
44 Tang Z, Bharadwaj R, Li B, Yu H: Mad2-independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. Dev Cell 2001;1:227–237.
45 Taylor SS, Ha E, McKeon F: The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. J Cell Biol 1998;142:1–11.
46 Fang G: Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. Mol Biol Cell 2002;13:755–766.
47 Hardwick KG, Johnston RC, Smith DL, Murray AW: *MAD3* encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p. J Cell Biol 2000;148: 871–882.
48 Sudakin V, Chan GK, Yen TJ: Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. J Cell Biol 2001;154:925–936.
49 Farr KA, Hoyt MA: Bub1p kinase activates the *Saccharomyces cerevisiae* spindle assembly checkpoint. Mol Cell Biol 1998;18:2738–2747.
50 Hardwick KG, Murray AW: Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. J Cell Biol 1995;131:709–720.
51 Brady DM, Hardwick KG: Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. Curr Biol 2000;10:675–678.
52 Seeley TW, Wang L, Zhen JY: Phosphorylation of human MAD1 by the BUB1 kinase in vitro. Biochem Biophys Res Commun 1999;257:589–595.
53 Tang Z, Shu H, Oncel D, Chen S, Yu H: Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. Mol Cell 2004;16:387–397.
54 Taylor SS, Hussein D, Wang Y, Elderkin S, Morrow CJ: Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. J Cell Sci 2001;114:4385–4395.
55 Chen RH: Phosphorylation and activation of Bub1 on unattached chromosomes facilitate the spindle checkpoint. EMBO J 2004;23:3113–3121.
56 Yamaguchi S, Decottignies A, Nurse P: Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint. EMBO J 2003;22:1075–1087.
57 Howell BJ, Moree B, Farrar EM, Stewart S, Fang G, Salmon ED: Spindle checkpoint protein dynamics at kinetochores in living cells. Curr Biol 2004;14:953–964.
58 Lopes CS, Sunkel CE: The spindle checkpoint: from normal cell division to tumorigenesis. Arch Med Res 2003;34:155–165.
59 Cahill DP, Lengauer C, Yu J, Riggins GJ, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B: Mutations of mitotic checkpoint genes in human cancers. Nature 1998;392:300–303.
60 Schlessinger J: Common and distinct elements in cellular signaling via EGF and FGF receptors. Science 2004;306:1506–1507.
61 Reis RM, Nakamura M, Masuoka J, Watanabe T, Colella S, Yonekawa Y, Kleihues P, Ohgaki H: Mutation analysis of *hBUB1*, *hBUBR1* and *hBUB3* genes in glioblastomas. Acta Neuropathol 2001;101:297–304.
62 Hernando E, Orlow I, Liberal V, Nohales G, Benezra R, Cordon-Cardo C: Molecular analyses of the mitotic checkpoint components *hsMAD2*, *hBUB1* and *hBUB3* in human cancer. Int J Cancer 2001;95:223–227.
63 Yamaguchi K, Okami K, Hibi K, Wehage SL, Jen J, Sidransky D: Mutation analysis of *hBUB1* in aneuploid HNSCC and lung cancer cell lines. Cancer Lett 1999;139:183–187.
64 Shigeishi H, Yokozaki H, Kuniyasu H, Nakagawa H, Ishikawa T, Tahara E, Yasui W: No mutations of the Bub1 gene in human gastric carcinomas. Oncol Rep 2001;8:791–794.
65 Langerod A, Stromberg M, Chin K, Kristensen VN, Borresen-Dale AL: *BUB1* infrequently mutated in human breast carcinomas. Hum Mutat 2003;22:420.

66 Jaffrey RG, Pritchard SC, Clark C, Murray GI, Cassidy J, Kerr KM, Nicolson MC, McLeod HL: Genomic instability at the *BUB1* locus in colorectal cancer, but not in non-small cell lung cancer. Cancer Res 2000;60:4349–4352.
67 Ouyang B, Knauf JA, Ain K, Nacev B, Fagin JA: Mechanisms of aneuploidy in thyroid cancer cell lines and tissues: evidence for mitotic checkpoint dysfunction without mutations in *BUB1* and *BUBR1*. Clin Endocrinol 2002;56:341–350.
68 Gemma A, Seike M, Seike Y, Uematsu K, Hibino S, Kurimoto F, et al: Somatic mutation of the *hBUB1* mitotic checkpoint gene in primary lung cancer. Genes Chromosomes Cancer 2000;29:213–218.
69 Ru HY, Chen RL, Lu WC, Chen JH: hBUB1 defects in leukemia and lymphoma cells. Oncogene 2002;21:4673–4679.
70 Hempen PM, Kurpad H, Calhoun ES, Abraham S, Kern SE: A double missense variation of the *BUB1* gene and a defective mitotic spindle checkpoint in the pancreatic cancer cell line Hs766T. Hum Mutat 2003;21:445.
71 Sato M, Sekido Y, Horio Y, Takahashi M, Saito H, Minna JD, Shimokata K, Hasegawa Y: Infrequent mutation of the *hBUB1* and *hBUBR1* genes in human lung cancer. Jpn J Cancer Res 2000;91:504–509.
72 Imai Y, Shiratori Y, Kato N, Inoue T, Omata M: Mutational inactivation of mitotic checkpoint genes, *hsMAD2* and *hBUB1*, is rare in sporadic digestive tract cancers. Jpn J Cancer Res 1999;90: 837–840.
73 Michel LS, Liberal V, Chatterjee A, Kirchwegger R, Pasche B, Gerald W, et al: *MAD2* haploinsufficiency causes premature anaphase and chromosome instability in mammalian cells. Nature 2001;409:355–359.
74 Dai W, Wang Q, Liu T, Swamy M, Fang Y, Xie S, et al: Slippage of mitotic arrest and enhanced tumor development in mice with *BubR1* haploinsufficiency. Cancer Res 2004;64:440–445.
75 Lee H, Trainer AH, Friedman LS, Thistlethwaite FC, Evans MJ, Ponder BA, Venkitaraman AR: Mitotic checkpoint inactivation fosters transformation in cells lacking the breast cancer susceptibility gene, *Brca2*. Mol Cell 1999;4:1–10.
76 Shichiri M, Yoshinaga K, Hisatomi H, Sugihara K, Hirata Y: Genetic and epigenetic inactivation of mitotic checkpoint genes *hBUB1* and *hBUBR1* and their relationship to survival. Cancer Res 2002;62:13–17.
77 Doak SH, Jenkins GJ, Parry EM, Griffiths AP, Baxter JN, Parry JM: Differential expression of the MAD2, BUB1 and HSP27 genes in Barrett's oesophagus – their association with aneuploidy and neoplastic progression. Mutat Res 2004;547:133–144.
78 Myrie KA, Percy MJ, Azim JN, Neeley CK, Petty EM: Mutation and expression analysis of human BUB1 and BUB1B in aneuploid breast cancer cell lines. Cancer Lett 2000;152:193–199.
79 Wang RH, Yu H, Deng CX: A requirement for breast-cancer-associated gene 1 (*BRCA1*) in the spindle checkpoint. Proc Natl Acad Sci USA 2004;101:17108–17113.
80 Hernando E, Nahle Z, Juan G, Diaz-Rodriguez E, Alaminos M, Hemann M, et al: Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature 2004;430:797–802.
81 Hanks S, Coleman K, Reid S, Plaja A, Firth H, Fitzpatrick D, et al: Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in *BUB1B*. Nat Genet 2004;36:1159–1161.

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Protein Kinases That Regulate Chromosome Stability and Their Downstream Targets

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Abstract

Upon genotoxic stress, checkpoint machinery in eukaryotic cells induces cell-cycle arrest, thus allowing the cells to repair damaged DNA or stalled replication forks. The checkpoint machinery is mediated by phosphorylation cascades involving protein kinases and their target proteins. Since the genome is under constant threat from DNA damage due to radiation, chemicals and replication errors, checkpoint dysregulation can cause catastrophic DNA damage, resulting in chromosome instability, aneuploidy, and even tumorigenesis. Two parallel pathways that respond to DNA-damage stress have been extensively studied. The first is the ATM pathway, which responds to double-stranded DNA breaks, while the second is the ATR pathway, which primarily responds to agents that interfere with normal DNA replication. The ATM and ATR kinases activate their downstream target proteins by phosphorylating specific serine or threonine residues. Dephosphorylation by protein phosphatase (PP2A) also participates in the regulation of these phosphorylation signals. Of the target proteins, the two effector kinases CHK1 and CHK2 are particularly important because they phosphorylate additional substrates to maintain chromosome stability after various DNA damaging insults. Recent observations indicate that other protein kinases that control centrosome duplication and chromosome segregation during the cell cycle also play essential roles in maintaining genomic stability.

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Abbreviations

ARF, alternative reading frame; A-T, ataxia-telangiectasia; ATM, ataxia telangiectasia mutated; ATR, ATM–Rad3-related; ATRIP, ATR-interacting protein; BAD, Bcl-2/Bcl-XL-antagonist causing cell death; BASC, BRCA1-associated genome surveillance complex; BLM1, Bloom's syndrome mutated 1;

BRCA1, familial breast and ovarian cancer locus 1; BS, Bloom's syndrome; CDC25, cell division cycle 25; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; DSBs, double-strand breaks; E2F, early gene 2 factor; FA, Fanconi's anemia; FANCD2, Fanconi anemia complementation group D2; FAT, FRAP ATM and TRRAP; FATC domain, FAT at C-terminal domain; H2AX, histone H2A variant X; IR, ionizing radiation; LATS2, large tumor suppressor 2; MDC1, mediator of DNA damage checkpoint protein 1; MCM2, minichromosome maintenance 2; MPS1, monopolar spindle 1; MRE11, meiotic recombination 11; MRN complex, MRE11-RAD50-NBS1 complex; NBS1, Nijmegen breakage syndrome gene 1; OA, ocadaic acid; PCNA, proliferating cell nuclear antigen; PI3-K, phosphatidylinositol 3-kinase; PLK, polo-like kinase; PML, promyelocytic leukaemia; PP2A, protein phosphatase 2A; PP5, protein phosphatase 5; PTEN, phosphatase and tensin homolog; RAD, Radiation; RB, retinoblastoma; RFC, replication factor C; RPA, replication protein A; SMC1, structural maintenance of chromosomal protein 1; TLK, Tousled-like kinase; TopBP1, DNA topoisomerase II beta-binding protein 1; TTK, tyrosine or serine/threonine kinase; UV, ultraviolet light; 53BP1, p53-binding protein 1.

Chromosomal instability, which can be manifested as multiple alterations in chromosome number and structure, is an important feature of tumor cells and a leading cause of human cancer [1]. Chromosomal instability is caused by defects in DNA damage repair, cell-cycle checkpoint regulation, chromosomal segregation during mitosis, centrosome regulation, and telomere maintenance [2–11]. These abnormalities impair the precise mitotic distribution of the genome and generate various types of chromosomal imbalances in the newly produced daughter cells [1–11]. To cope with the DNA-damaging agents that can generate these injuries, eukaryotic cells have evolved various regulatory systems that are connected to a complex network of cellular signaling pathways. For example, some checkpoint signaling pathways are activated by genotoxic insults: these systems then block cell cycle progression, trigger apoptosis, or induce DNA repair [1–3, 11]. Some DNA damage signals are propagated by a phosphorylation cascade that involves protein kinases and their target proteins [1, 5]. It is known at present that two major parallel pathways respond to DNA-damaging stresses, namely, the ATM-CHK2 and ATR-CHK1 pathways [1, 4–7, 8, 9, 12–15]. These pathways are known to play a primary role in transmitting phosphorylation signals to downstream targets [1, 5]. ATM (ataxia telangiectasia mutated) is situated at the top of a signaling cascade that responds to double-strand breaks (DSBs) of DNA and plays a central role in coordinating the resulting cellular responses [4–7]. In contrast, ATR (ATM–Rad3-related) initiates the signaling pathway activated by stresses that induce a replication-type insult, such as hydroxyurea treatment, ultraviolet light (UV) and hypoxia [7, 13]. Since human cells activate an ATR/ATM-regulated

DNA-damage response network to delay or prevent cancer early in tumorigenesis, mutations in this network appear to promote genomic instability and tumor progression [3–8]. In this review, I will focus on the recent progress made in understanding how these pathways respond to diverse genotoxic stresses in mammals.

ATM Kinase

The human autosomal recessive disorder ataxia-telangiectasia (A-T) is characterized by progressive neurodegeneration, immunodeficiency and a predisposition to tumorigenesis [1, 4–7], and cells from A-T patients often display chromosome aberrations after exposure to radiation [1, 4–7]. ATM, the product of the gene mutated in A-T, plays a central role in the recognition and signaling of DSBs that occur in cells exposed to ionizing radiation (IR) or radiomimetic chemicals [4–7]. ATM is activated in response to DNA damage and initiates cellular signaling pathways that reduce chromosomal breakage, thus aiding cell survival. *Atm*-deficient mice are viable but display retarded growth and severe defects in T-cell maturation that lead to the development of thymomas, while *Atm*-deficient fibroblasts exhibit high levels of DSBs [16].

ATM is a member of the phosphatidylinositol 3-kinase (PI3-K) family and phosphorylates target proteins that trigger the G_1/S, G_2/M and S phase checkpoints, thereby arresting cell cycle progression until the lesions are properly repaired [2, 6]. The FAT (FRAP, ATM, and TRRAP) and FATC (FAT at C-terminal) domains immediately adjacent to the C-terminally located kinase domain of ATM are important for its kinase activity. In unirradiated cells, ATM is kept inactive by dimerization, wherein the kinase domain binds to the FAT domain of another ATM molecule (fig. 1). Radiation stimulates the intermolecular autophosphorylation of ATM on its Ser1981 residue, which induces the dissociation of the dimer; this activates the kinase property of ATM, which then initiates events involved in the cellular response to radiation [1, 5, 6, 17]. Appropriate dephosphorylation of ATM by protein phosphatase 2A (PP2A) also plays a pivotal role in its functions since inhibition of a PP2A-like protein phosphatase activity by the specific inhibitor ocadaic acid (OA) induces the rapid phosphorylation of ATM on Ser1981. This suggests that PP2A normally maintains ATM in an inactive state [18]. Indeed, ATM associates constitutively with the scaffolding and catalytic subunits of PP2A (PP2A-A and PP2A-C, respectively) in vivo, and exposure to IR causes a rapid, phosphorylation-dependent disruption of the interaction between ATM and PP2A. PP2A-mediated dephosphorylation seems to function as a feedback mechanism to silence ATM activity since PP2A activity is regulated by its association partner Cyclin G1, which is induced by DNA damage in a p53-dependent manner (p53 is a target

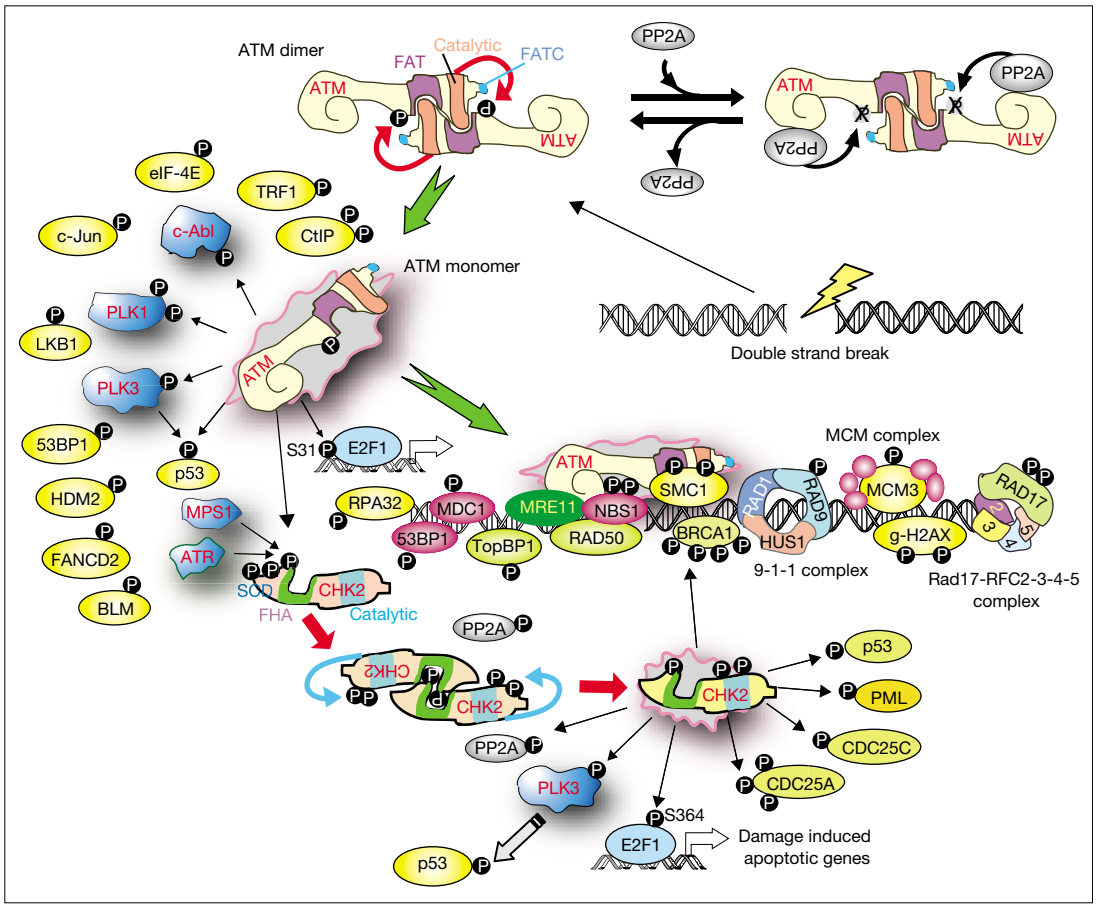

Fig. 1. A model showing the activation of ATM by DSBs and the subsequent phosphorylation cascade. Also shown are the downstream phosphorylation targets of ATM (their phosphorylation sites are listed in table 1). These phosphorylated proteins further propagate the signal to the downstream targets, thereby inducing various cellular events. Protein kinases are highlighted by shading and their names are shown in red. ATM forms an inactive dimer in non-irradiated cells due to the binding of its catalytic domain to the FAT domain of another ATM molecule. Radiation-induced DSBs induce the intermolecular autophosphorylation of the ATM dimer on Ser1981. This causes the ATM dimer to dissociate into ATM monomers, which activates their kinase function. The ATM molecules then phosphorylate the target proteins displayed in the figure and thereby initiate the cellular responses to irradiation. In non-irradiated cells, PP2A inhibits ATM activation by dephosphorylating the Ser1981 residue. CHK2, a target of ATM, is activated by phosphorylation at Thr68 that is mediated primarily by ATM. This induces CHK2 dimerization and subsequent intermolecular autophosphorylation events. This activates the CHK2 kinase and induces it to phosphorylate its target proteins. ATR or MPS1 can also phosphorylate CHK2 at Thr68. ATM also activates other protein kinases by phosphorylation but the consequences of this remain unclear.

of ATM) and plays a role in regulation of p53 degradation by associating with MDM2 complexes containing ARF or PP2A [1, 19]. Protein phosphatase 5 (PP5) also interacts with ATM to activate the phosphorylation of ATM substrates and the autophosphorylation of ATM on Ser1981, indicating that PP5 plays a role in ATM-mediated DNA damage-induced signaling [20].

To date, many phosphorylation targets of ATM have been identified (table 1). Their characteristics indicate that ATM is involved not only in the DNA damage response but also in other subcellular events, many of which are involved in maintaining chromosomal stability [1, 5, 6, 16]. The ATM phosphorylation targets include NBS1 (Nijmegen breakage syndrome gene 1) [21] and MRE11 (meiotic recombination 11). These are components of the MRN (MRE11/RAD50/NBS1) complex that acts as a DSB sensor for ATM and recruits ATM to broken DNA molecules, thereby leading to the phosphorylation of the downstream cellular target CHK2 (checkpoint kinase 2) [1, 7, 22, 23]. It appears that ATM activates MRN to unwind the DNA ends and generate single stranded DNA. Notably, MRN complex may not only be a downstream effector of ATM but also may function in activating ATM to initiate phosphorylation of cellular substrates [23], which suggests an existence of amplification loop in ATM activation. ATM autophosphorylation is not required for this MRN-induced monomerization of ATM. The MRN complex also recruits ATM targets such as BRCA1 (familial breast and ovarian cancer locus 1), which forms a larger complex called BASC (BRCA1-associated genome surveillance complex) [10]. Another important phosphorylation target of ATM is the histone H2A variant (H2AX) whose massive phosphorylation in the chromatin surrounding the DSB sites is important for retaining players like MRN at the lesion [24].

ATM also phosphorylates the budding yeast Rad9 orthologs, 53BP1 (p53-binding protein 1) and MDC1 (mediator of DNA damage checkpoint protein 1), which associate with IR-induced foci at sites of DNA repair and play essential roles in the DNA damage signaling pathway [1, 4, 6, 25, 26]. MDC1 is hyperphosphorylated by ATM in response to IR, after which it rapidly relocalizes to nuclear foci that subsequently come to contain the MRN complex, H2AX and 53BP1 [25–27]. Downregulation of MDC1 induces a radio-resistant DNA synthesis phenotype; thus, the MDC1-mediated formation of MRN complex foci at sites of DNA damage appears to be crucial for the activation of the intra-S-phase checkpoint [1, 2, 28]. ATM also phosphorylates BLM1 (Bloom's syndrome mutated 1), the RecQ helicase that is mutated in Bloom's syndrome (BS). BS is a rare human genetic disorder characterized by dwarfism, immunodeficiency, genomic instability and cancer predisposition [29]. Cells derived from BS patients show genomic instability due to an elevated frequency of sister-chromatid exchange. BLM1 is a component of BASC and forms a BLM pathway that is activated in response to both crosslinked DNA and replication fork stall. Fanconi

Table 1. Phosphorylation sites of proteins targeted by protein kinases and the cellular events they regulate

Kinase[a]	Target[a]	Phosphorylation site[b]	Cellular event that is regulated[c]
ATM	**ATM**	S1981	Self-activation
	BLM	T99	G_2/M checkpoint
	Artemis	S645	VDJ recombination
	BRCA1	S1387, S1423, S1457, S1524	GIDI
	c-Abl	S465	Apoptosis
	c-Jun	S63	Gene regulation
	CHK2	S33, S35, T68	G_1/S checkpoint/DSB repair
	CtIP	S664, S745	Transcription
	eIF-4E	S111	Translation initiation
	E2F1	S31	Transcription/Apoptosis
	FANCD2	S222	Intra-S checkpoint/DSB repair
	H2AX	S139, S140	IR-induced foci
	LKB1	T366	G_1/S checkpoint
	MCM3	S535	Replication/cell cycle
	MDC1	ND	IR-induced foci
	MRE11	ND	Intra-S checkpoint/DSB repair
	HDM2	S395	Apoptosis
	NBS1	S278, S343	Intra-S checkpoint/DSB repair
	p53	S15 (S9, S46)	Apoptosis/checkpoint
	PLK1	S137, T210*	Cell cycle
	PLK3	ND	Cell cycle
	RAD9	S272	DNA repair/checkpoint
	RAD17	S635, S645	DNA repair/checkpoint
	RPA32	T21	Intra-S checkpoint
	SMC1	S957, S966	Chromosome regulation
	TopBP1	S405	IR-induced foci
	TRF1	S219	Telomere dysfunction
	53BP1	S25	Apoptosis
ATR	ATRIP	S68, S72	ATR-activation
	BLM	T99	G_2/M checkpoint
	BRCA1	S1387, S1423, S1457, S1524	GIDI
	CHK1	S317, S345	Intra-S checkpoint
	CHK2	T68	G_1/S checkpoint/DSB repair
	E2F1	S31*	Transcription/Apoptosis
	H2AX	S139, S140	IR-induced foci
	MCM2	S108	Replication/cell cycle
	NBS1	S278, S343	Intra-S checkpoint
	p53	S15	Apoptosis/checkpoint
	Rad9[#]	T412/S423	DNA repair/checkpoint
	RAD17	S635, S645	DNA repair/checkpoint
	RPA	T21(?)	Intra-S checkpoint
	SMC1	S966	Chromosome regulation
	TopBP1	S405	IR-induced foci

Table 1. (continued)

Kinase[a]	Target[a]	Phosphorylation site[b]	Cellular event that is regulated[c]
CHK1	BAD	S155, (S170)	Apoptosis
	CDC25A	S75, S278	Cell cycle
	CDC25C	S216	Cell cycle
	Claspin	T916 (xT906), S945 (xS934)	Cell cycle
	p53	S20	Cell cycle/Apoptosis
	RelA (p65)	T505	Transcription/Apoptosis
	TLK	S695	Chromatin remodeling
CHK2	BRCA1	S988	Repair/Recombination
	CDC25A	S123, S178, S292	Cell cycle/Apoptosis
	CDC25C	S216	Cell cycle
	CHK2	T383, T387	Self-activation
	E2F	S364	Cell cycle/Transcription
	PML	S117	Cell cycle
	PLK3	ND	Cell cycle
	p53	S20	Apoptosis
PKB/AKT	**CHK1**	S280	Intra-S checkpoint
MPS1/TTK	**CHK2**	T68	G_1/S checkpoint/Repair

[a]Protein kinases are shown in bold; [#]: protein of the fission yeast *S. pombe*.
[b] *: see [5] for the references of these phosphorylation events; ND: not determined.
[c]GIDI: G_2M checkpoint/Intra-S checkpoint/DNA repair/IR-induced foci.

anemia (FA) is also a rare autosomal recessive genetic condition associated with bone marrow failure, reduced fertility, and a predisposition to cancer (especially myeloid leukemia) [10, 30]. FA-derived cells show chromosome instability, and hypersensitivity to agents that induce DNA interstrand crosslinks. FANCD2 (Fanconi anemia complementation group D2), an ATM substrate, is a downstream effector that is activated by the FA core complex, which is composed of FANC proteins (A, C, E, F, G and L). It colocalizes and co-immunoprecipitates with BLM1, suggesting that the BLM and FA pathways cooperate in response to DNA damage [31]. Monoubiquitination of FANCD2 is essential for its activity in DNA repair and tumor suppression; when cells exit S phase or recommence cycling after a DNA damage insult, a specific deubiquitinating enzyme USP1 deubiquitinates FANCD2, thus recycling it [32].

ATM also phosphorylates the components of DNA damage checkpoint sensors, including RAD (Radiation) 17 and RAD9. These phosphorylation events cause the formation of the RAD17-RFC (replication factor C) (2–5) checkpoint clamp loader, or the 9-1-1 (RAD9-HUS1-RAD1) complex that forms a clamp-like

structure that resembles PCNA (proliferating cell nuclear antigen) [1, 33]. These complexes help to detect DNA damage and initiate the signal transduction cascades that employ the CHK1 (checkpoint kinase 1) and CHK2 kinases (see below) and CDC25 (cell division cycle 25) phosphatase [5, 6, 9]. RAD9 knockdown reduces the phosphorylation of RAD17 and the cohesin SMC1 (structural maintenance of chromosomal protein 1) in response to DNA damage, which suggests the major role of RAD9 is in the activation of the S-phase checkpoint and the maintenance of chromosome stability [34]. There are two parallel ATM-dependent pathways that control the IR-induced S-phase checkpoint [1, 3]: one involves the phosphorylation of NBS1 and SMC1 while the other involves the phosphorylation of the downstream CHK2 kinase; both of these phosphorylation events elicit the secondary response phase that regulates G_2/M entry.

SMC1, a component of the cohesin complex, plays a role in promoting the repair of gaps and deletions. Thus, it is required for postreplicative DSB repair [35]. SMC1-null mammalian cells are inviable, and overexpression of a dominant-inhibitory SMC1 mutant abrogated the IR-induced S-phase checkpoint and caused radiosensitivity. Since defects in the ATM-induced phosphorylation of p53, NBS1, and BRCA1 have no significant effects on radiosensitivity, SMC1 seems to be the only ATM target that directly affects cell survival. The ATM-SMC1 pathway functions in response to DNA damage that is induced not only by IR but also by DNA-damaging agents such as hexavalent chromium [36]. Cells from the knock-in mouse in which the Ser957 and Ser966 residues of SMC1 (the sites phosphorylated by ATM) were substituted by alanines show normal phosphorylation and focus formation of the ATM, NBS1, and BRCA1 proteins after IR, but exhibited a defective S-phase checkpoint, decreased survival, and increased chromosomal aberrations after DNA damage [13]. Thus, SMC1 appears to be the critical downstream target of ATM in its DNA damage response pathway.

ATR

ATR (ATM and Rad3-related) also belongs to the PI3-K family. It has a C-terminal kinase domain and regions of homology to other PI3-K family members, which include ATM and DNA-dependent protein kinase [1, 7, 12, 13]. Unlike the *ATM* gene, however, the *ATR* gene is essential since null mutations in mice are embryonically lethal and cells derived from these mice are not viable. This may be why human diseases caused by mutations in the *ATR* gene have not been identified, apart from the autosomal recessive disorder ATR-Seckel syndrome, which is a subclass of Seckel syndrome arising from a point mutation in *ATR* gene [9, 13]. ATR-Seckel cells display impaired phosphorylation of ATR-dependent substrates and impaired G_2/M checkpoint arrest after

UV-induced replication stalling [37]. Unlike ATM, ATR is responsible for preventing the replication of damaged DNA and inhibiting entry into mitosis before the duplication of the genome is complete. ATR binds directly to DNA but does not recognize DSBs; rather, it prefers to bind to a 50-bp duplex containing DNA lesions generated by UV irradiation [9, 13]. Thus, ATR controls S-phase progression in response to DNA damage and replication fork stalling, including damage caused by UV irradiation, hypoxia, and replication inhibitors like aphidicolin and hydroxyurea [9, 13].

The role of ATR is also analogous to that of ATM and it phosphorylates many of the same proteins that are phosphorylated by ATM (table 1). Both ATR and ATM phosphorylate serine or threonine residues when they are followed by a glutamine (SQ/TQ sequences) [38]. ATR is a monomeric protein and it is activated by complexing with the 86-kDa protein ATRIP (ATR-Interacting Protein). ATRIP is required for ATR accumulation at intranuclear foci induced by DNA damage [39]. ATR-ATRIP binds and phosphorylates RPA (replication protein A) on ssDNA when ATRIP recruits ATR to RPA-bound single-strand or double-strand DNA (fig. 2) [40]. ATR also controls the proteins in the RAD17-Rfc(2–5) checkpoint clamp loader or the 9-1-1 sensor complex by phosphorylating RAD17 or RAD9, respectively. Both RPA and ATRIP are also required for the RAD17-dependent loading of the 9-1-1 complex onto damaged chromatin in cells not undergoing DNA replication. BRCA1, a phosphorylation target of ATR after UV irradiation and hydroxyurea exposure, is required before this signal is propagated to the downstream effector CHK1 (see below). The BRCT domain-containing protein TopBP1 (DNA topoisomerase II beta-binding protein 1) also serves to mediate interactions between the checkpoint pathway and elements of the DNA repair and replication machinery. In fission yeast, $Rad3^{Sp}$ (the ATR ortholog of *S. pombe*) phosphorylates $Rad9^{Sp}$ on Thr412/Ser423; this phosphorylated form alone can associate with a two BRCT-domain region of the essential protein $Rad4^{Sp}$ (the TopBP ortholog) to activate the $Chk1^{Sp}$ damage checkpoint [41].

H2AX phosphorylation by ATR appears to occur after UV irradiation or inhibition of DNA replication, and serves to recruit chromatin-remodeling factors that alter chromatin accessibility. However, both downstream factors of H2AX, namely, 53BP1 and MDC1, are required for the recruitment of ATR to DNA damage sites, and the recruitment of MDC1 and/or 53BP1 is independent of ssDNA generation and RPA coating [25–27]. Thus, the generation of ssDNA and the recruitment of MDC1 and 53BP1 by gamma-H2AX (the phosphorylated form of H2AX) are independent events. SMC1 is phosphorylated on both Ser957 and Ser966 residues by ATM following IR-induced DNA damage, but upon exposure of cells to aphidicolin, ATR phosphorylates SMC1 on Ser966 but not on Ser957; Ser966-phosphorylated SMC1 is involved in fragile site expression [42].

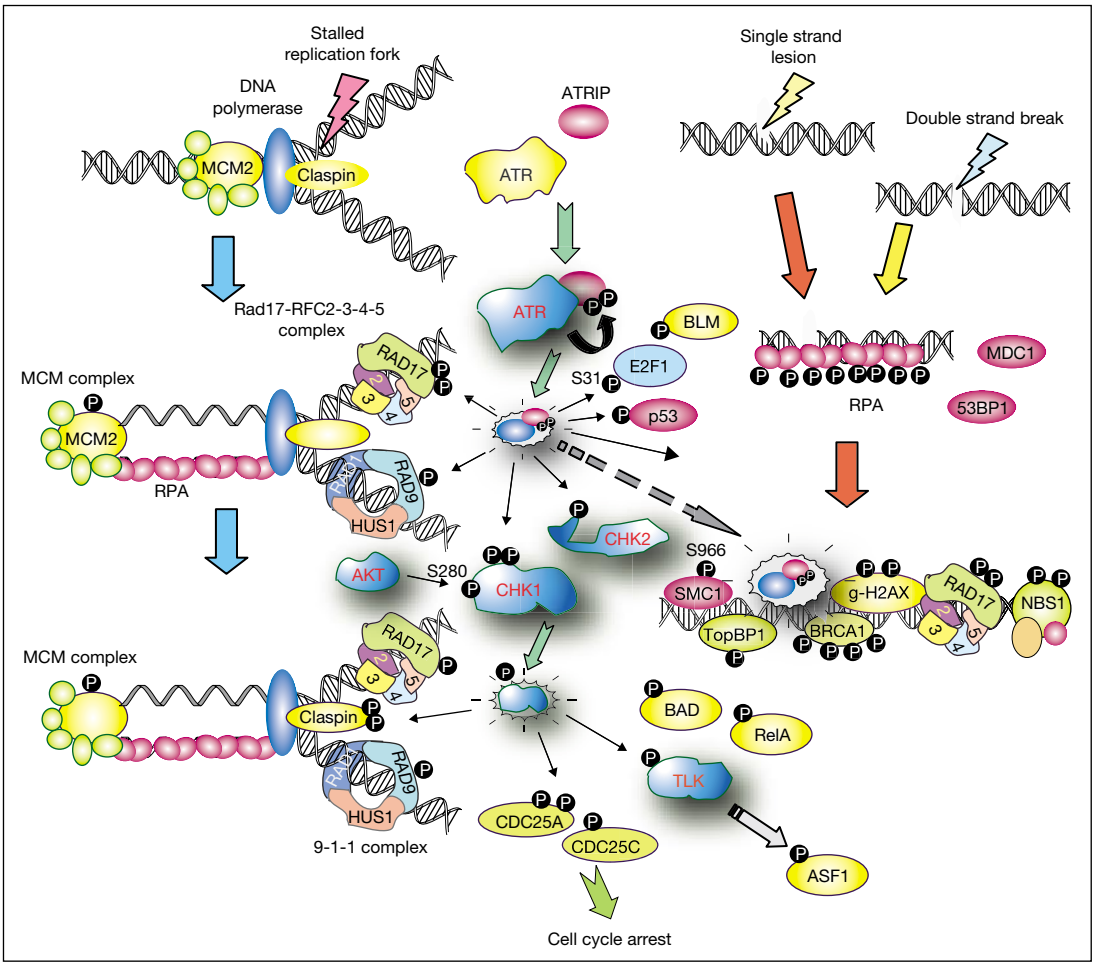

Fig. 2. A model showing the activation of ATR by a stalled replication fork, DSBs or single strand DNA lesions and the subsequent phosphorylation cascade. Also shown are the downstream phosphorylation targets of ATR (their phosphorylation sites are listed in table 1). ATR is a monomeric protein and its activation is regulated by ATRIP, an association partner and a phosphorylation target of ATR. Protein kinases are highlighted by shading and their names are given in red. CHK1 is activated by phosphorylation on Ser317 and Ser345 by ATR. It then phosphorylates its target proteins. One of these is TLK, which is also a protein kinase. This CHK1-mediated phosphorylation event activates it, after which it phosphorylates its own target protein ASF1, thereby propagating the signal downstream and eliciting the relevant cellular responses to DNA damage. PKB/AKT can also phosphorylate CHK1 but the site it phosphorylates (Ser280) is distinct from those targeted by ATR (Ser317 and Ser354).

In response to DNA damage and the stalling of replication forks, ATR also phosphorylates MCM2 (minichromosome maintenance 2) (on Ser108), a component of the MCM2–7 helicase complex that functions to initiate and elongate replication forks [43]. In response to hypoxia, ATR re-localizes to form nuclear foci and has been shown to phosphorylate many of its known downstream target molecules there, including NBS1, RAD17, CHK1, p53 and H2AX [12]. NBS1 also appears to facilitate ATR-dependent phosphorylation because NBS cell lines show a similar defect in ATR phosphorylation of CHK1 in response to UV irradiation- and HU-induced replication stalling [44]. A component of the tRNA synthetase complex, p18, directly interacts with ATM/ATR in response to DNA damage and behaves as a key factor for ATM/ATR-mediated p53 activation [45].

CHK1

CHK1 is an evolutionarily conserved protein kinase and a phosphorylation target of ATR (fig. 2). It was identified on the basis of its homology with fission yeast Chk1, which plays a pivotal role in signal transduction that regulates checkpoints in response to DNA damage [14]. In mammalian cells, the UV-damage signal that is sensed by ATR-ATRIP is transduced by CHK1, whereas the DSB signal that is sensed by ATM is transduced by CHK2 (see below), although there is some cross talk between these two pathways. CHK1 phosphorylates the CDC25A or CDC25C phosphatases to induce transient arrest in the G_1, S, and G_2 phases of the cell cycle [14]. CHK1 knockout is embryonically lethal in mice, and $CHK1^{-/-}$ ES cells treated with IR, UV or the replication inhibitor aphidicolin fail to delay entry into mitosis due to impaired G_2/M checkpoint regulation.

The phosphorylation targets of CHK1 (table 1) include Claspin, a CHK1-binding protein with marginal sequence homology to yeast Mrc1. It has been shown to be required for the ATR/ATM-dependent and -independent phosphorylation of CHK1 [46, 47]. It was shown that activated ATR-ATRIP only weakly phosphorylates CHK1 in a cell-free reaction using *Xenopus* extracts when Claspin is absent, whereas the addition of Claspin enhances the phosphorylation of CHK1 by not only ATR-ATRIP but also by CHK1 itself [48]. The Claspin homolog Mrc1 of the fission yeast *S. pombe* is a replication checkpoint adaptor protein that allows the sensor kinase $Rad3^{Sp}$-$Rad26^{Sp}$ (ATR-ATRIP) to activate the effector kinase $Cds1^{Sp}$ (CHK2); the DNA-binding domain of $Mrc1^{Sp}$ interacts preferentially with branched DNA structures [49]. Human Claspin, which has a ring-like structure, also binds with high affinity to branched DNA molecules [50]. The DNA-dependent binding of CHK1 to Claspin requires that the Thr916 and Ser945 residues, which lie within the

CHK1-binding domain of Claspin, are phosphorylated [51]. In *Xenopus*, Claspin becomes phosphorylated on Thr906, which creates a docking site for Plx1 (Polo-like *Xenopus* kinase 1, see below); this interaction promotes the phosphorylation of Claspin on a nearby serine (Ser934) by Plx1 [52]. Claspin also binds to BRCA1 and controls the phosphorylation of BRCA1 on Ser1524, a site whose phosphorylation is controlled by the ATR pathway; BRCA1 and Claspin then function to activate CHK1 [53].

In an ATR-dependent manner, CHK1 phosphorylates Thr505 of the NF-κB subunit RelA(p65), whose site is required for the ARF (alternative reading frame)-dependent repression of RelA transcriptional activity [54]. CHK1 also binds and phosphorylates the pro-apoptotic protein BAD (Bcl-2/Bcl-XL-antagonist, causing cell death) at Ser155 and Ser170 in vitro; the Ser155 phosphorylation is increased upon DNA damage but its physiological role remains elusive [55]. Notably, the Timeless protein that is essential for the viability and maintenance of the circadian rhythm interacts with CHK1 and the ATR-ATRIP complex, which play an important role in the regulation of replication and intra-S checkpoints. This suggests that there is a link between the circadian cycle and the DNA damage checkpoint response [56]. Protein kinase B/AKT (PKB/AKT) phosphorylates CHK1 on Ser280 in vivo, which reduces the ability of CHK1 to be activated by phosphorylation at another residue (Ser345) by ATM/ATR in response to DNA damage: it thereby blocks the entry of CHK1 into the ATR complex after replication arrest [57]. CHK1 function is also modulated by the tumor suppressor PTEN (phosphatase and tensin homolog), a phosphatase that dephosphorylates both protein and phosphoinositide substrates. Cells lacking PTEN show elevated levels of CHK1 phosphorylated on Ser345 [58]. Thus, loss of PTEN and the subsequent activation of PKB/AKT impair CHK1 activity through dephosphorylation on Ser345 or phosphorylation on Ser280, respectively, which results in the ubiquitination and reduced nuclear localization of CHK1; this ultimately promotes the genomic instability observed in PTEN-defective tumor cells [57, 58].

TLK (Tousled-like kinase) 1 and 2 are active during S phase of the cell cycle, and their kinase activities are rapidly suppressed in response to replication block and UV-induced DNA damage. Although it is CHK1 but not ATM that directly phosphorylates TLK, the downregulation of TLK activity after UV and replication block is ATM-dependent but ATR-independent, and NBS1 is required as an adaptor or scaffold in this ATM/TLK pathway [59]. This suggests the existence of an ATM/CHK1/TLK pathway [60]. TLKs phosphorylate ASF1 (anti-silencing function 1), a histone chaperone involved in replication-dependent chromatin assembly, during S phase when TLKs are maximally active to regulate chromatin assembly during DNA replication and nuclear division [61].

CHK2

CHK2 is activated upon DNA damage by phosphorylation on Thr68 by ATM (fig. 1). It then phosphorylates downstream targets to cause cell cycle arrest and apoptosis [15]. ATR may similarly phosphorylate CHK2 after high levels of DNA damage. The CHK2 protein consists of three distinct functional domains, namely, an SQ/TQ cluster domain (SCD), a forkhead associated (FHA) domain, and a Ser/Thr kinase domain. CHK2 exists as a monomer in unperturbed cells, but after DNA damage, its FHA domain binds to a region of another CHK2 molecule that spans the phosphorylated Thr68 residue. The resulting dimer is then subjected to intermolecular phosphorylations on Thr383 and Thr387 and autophosphorylation of the Ser516 residue within the kinase domain; this results in kinase activation [15]. MDC1 also selectively associates with the phospho-Thr68 residue on CHK2 through its FHA domain, which suggests that this phospho-Thr68 plays a role in other checkpoint responses. CHK2 associates with and phosphorylates the B′ gamma 3 regulatory subunit of PP2A, which enhances the catalytic phosphatase activity of PP2A; this suggests that PP2A-mediated phosphorylation may also participate in the ATM-CHK2 pathway [62]. Unlike CHK1, $CHK2^{-/-}$ mice are viable and appear to exhibit near-normal checkpoint responses; however, in humans, mutations in CHK2 cause the cancer-prone Li-Fraumeni-like syndrome [8].

One of the phosphorylation targets of CHK2 (table 1) is the PML (promyelocytic leukemia) tumor-suppressor protein, which is the major structural component of the PML-NB (nuclear body). PML plays a pivotal role in regulating the apoptosis that is induced by DNA damage and in maintaining chromosome stability; both functions are mediated by the ability of PML to potentiate p53 function [63]. Upon DNA damage, ATM and CHK2 (the latter can phosphorylate PML on Ser117) localize to PML NBs. PML NBs are generally present in all mammalian cells and serve as depots for DNA repair and checkpoint proteins, including ATM, ATR, and CHK2 [64]. They also represent sites of post-translational modification that regulate DNA repair, transcription, apoptosis, tumor suppression, cellular senescence and viral pathogenicity. Nonetheless, PML is dispensable because PML knockout mice are viable, although they do exhibit an increased incidence of tumors in response to carcinogens. Notably, the PML isoform PML3 localizes to centrosomes and its deficiency leads to dysregulation of the centrosome duplication checkpoint, causing centrosome amplification and chromosome instability [65].

The transcription factor E2F1 (early gene 2 factor), a critical downstream target of the tumor suppressor RB (retinoblastoma), regulates the expression of several hundred genes that are involved not only in DNA replication and cell cycle progression but also in DNA damage repair, apoptosis, differentiation and development

[66]. Upon severe DNA damage, E2F1 directs the cell towards apoptosis rather than towards growth arrest by inducing the expression of the ATM and CHK2 genes, which ultimately stabilizes p53 [67]. E2F1 is phosphorylated by ATM/ATR (Ser31) and CHK2 (Ser364) (table 1), which leads to E2F1 accumulation and induction of apoptosis [68]. The subsequent activation of E2F1 leads to the phosphorylation of p53 on its Ser46 residue; this modification is important for the cooperation between E2F1 and p53 that leads to apoptosis [69]. E2F1 also controls the induction of p73, a p53-related transcription factor, in response to DNA damage; this mediates the p53-independent cell death produced by cytotoxic drugs [70]. 14-3-3 tau, a phosphoserine-binding protein, interacts with ATM-phosphorylated E2F1 during DNA damage and inhibits E2F1 ubiquitination, thereby stabilizing E2F1. 14-3-3 tau is also required for the expression and induction of the pro-apoptotic targets of E2F1, such as p73, Apaf-1, and caspases, during DNA damage [71].

Other Kinases

The four mammalian polo-like kinase (PLK) family members (PLK1, 2, 3, or 4) are critical regulators of cell cycle progression, mitosis, cytokinesis, and the DNA damage response, and their dysregulation causes genomic instability [72]. PLK1 interacts with CHK2, colocalizes with CHK2 at centrosomes, and can phosphorylate CHK2 on Thr68 [60]. PLK3 also interacts with CHK2 and the two proteins can mutually phosphorylate one another. The phosphorylation of PLK1 at Ser137 and Thr210 during mitosis may be mediated by ATM/ATR. This is inhibited by DNA damage in asynchronous cells but not in mitotic cells [73]. Budding yeast cells that are unable to repair a DSB ultimately escape the DNA damage checkpoint arrest and enter mitosis, which is called 'adaptation' and depends on yeast PLK (Cdc5). *Xenopus* extracts can also adapt to a long-lasting stall in DNA replication, with Plx1 (*Xenopus* PLK1) being required for cell cycle reentry following a DNA damage-induced arrest [74]. Moreover, Claspin is essential for the ATR-dependent activation of CHK1 in *Xenopus* egg extracts; it becomes phosphorylated on Thr906, which creates a docking site for Plx1 during this checkpoint response [50]. This interaction promotes the phosphorylation of Claspin on Ser934 by Plx1; when Claspin dissociates from chromatin and CHK1 becomes inactivated, this results in 'adaptation' and entry into mitosis despite the presence of incompletely replicated DNA.

MPS1 (monopolar spindle 1), a kinase required for normal mitotic progression, centrosome duplication and cytokinesis, has recently been suggested to be a critical regulator of genomic stability [75]. TTK (tyrosine or serine/threonine kinase), a human homolog of yeast MPS1 that regulates the spindle assembly checkpoint, is a CHK2-interacting protein. It directly phosphorylates

CHK2 on Thr68. The expression of a TTK kinase-dead mutant interferes with IR- or UV-induced G_2/M arrest, suggesting possible cross talk between the spindle assembly checkpoint and the DNA damage checkpoint [76]. Centrosomes are involved in the regulation of cell cycle regulatory events such as the monitoring of interphase microtubule arrays responsible for cell polarity and the mitotic spindle, the bipolar separation of chromosomes during mitosis, entry into mitosis, cytokinesis, G_1/S transition and DNA damage [77]. The rapidly growing list of centrosome-associated regulatory proteins include p53, BRCA1, CHK1, CHK2, TopBP1, Aurora-A, LATS2 (large tumor suppressor 2) and PLK1, which suggests there is a link between centrosome function and the DNA damage response. For example, ATR-Seckel cells display an increased number of centrosomes in mitotic cells, suggesting a novel role for ATR in regulating centrosome stability [36]. Aurora-A phosphorylates LATS2 on Ser83, and inhibition of this phosphorylation perturbs its centrosomal localization [78]; this suggests the existence of another phosphorylation cascade that is involved in regulating centrosome duplication and chromosome stability.

Concluding Remarks and Future Perspectives

The number of players in the ATM- and/or ATR-mediated phosphorylation signaling in response to DNA damage is increasing, which helps us to understand the mechanism how cells respond to DNA damaging stress. Nonetheless, much less is known about the events in the downstream of ATM/CHK2 or ATR/CHK1 signaling pathways, where other uncharacterized protein kinases are activated to phosphorylate their target proteins, resulting in subsequent subcellular events. Moreover, the detailed mechanism on the regulation of dephosphorylation of these targeted sites remains elusive, although it is almost certain that protein phosphatases will play important roles in many aspects of the ATM/ATR signaling. Future studies of these regulatory mechanisms and modulation of their activities might offer some future benefit to the development of cancer therapeutics.

References

1 Nojima H: G1 and S-phase checkpoints, chromosome instability, and cancer; in Schoenthal AH (ed.): Methods in Molecular Biology, Checkpoint Controls and Cancer – Methods and Protocols. Humana Press, Totowa NJ, 2004, vol. 1, pp 3–49.
2 Kastan MB, Bartek J: Cell-cycle checkpoints and cancer. Nature 2004;432:316–323.
3 Bartek J, Lukas C, Lukas J: Checking on DNA damage in S phase. Nat Rev Mol Cell Biol 2004;5:792–804.
4 McKinnon PJ: ATM and ataxia telangiectasia. EMBO Rep 2004;5:772–776.
5 Kurz EU, Lees-Miller SP: DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair (Amst) 2004;3:889–900.

6 Lavin MF, Birrell G, Chen P, Kozlov S, Scott S, Gueven N: ATM signaling and genomic stability in response to DNA damage. Mutat Res 2005;569:123–132.
7 Koundrioukoff S, Polo S, Almouzni G: Interplay between chromatin and cell cycle checkpoints in the context of ATR/ATM-dependent checkpoints. DNA Repair (Amst) 2004;3:969–978.
8 Motoyama N, Naka K: DNA damage tumor suppressor genes and genomic instability. Curr Opin Genet Dev 2004;14:11–16.
9 Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S: Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 2004;73:39–85.
10 Venkitaraman AR: Tracing the network connecting BRCA and Fanconi anaemia proteins. Nat Rev Cancer 2004;4:266–276.
11 Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J, Bartek J: DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–870.
12 Hammond EM, Giaccia AJ: The role of ATM and ATR in the cellular response to hypoxia and re-oxygenation. DNA Repair (Amst) 2004;3:1117–1122.
13 Shechter D, Costanzo V, Gautier J: Regulation of DNA replication by ATR: signaling in response to DNA intermediates. DNA Repair (Amst) 2004;3:901–908.
14 Chen Y, Sanchez Y: Chk1 in the DNA damage response: conserved roles from yeasts to mammals. DNA Repair (Amst) 2004;3:1025–1032.
15 Ahn J, Urist M, Prives C: The Chk2 protein kinase. DNA Repair (Amst) 2004;3:1039–1047.
16 Barlow C, Hirotsune S, Paylor R, Liyanage M, Eckhaus M, Collins F, Shiloh Y, Crawley JN, Ried T, Tagle D, Wynshaw-Boris A: Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell 1996;86:159–171.
17 Bakkenist CJ, Kastan MB: DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421:499–506.
18 Goodarzi AA, Jonnalagadda JC, Douglas P, Young D, Ye R, Moorhead GB, Lees-Miller SP, Khanna KK: Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. EMBO J 2004;23:4451–4461.
19 Kimura SH, Nojima H: Cyclin G1 associates with MDM2 and regulates accumulation and degradation of p53 protein. Genes Cells 2002;7:869–880.
20 Ali A, Zhang J, Bao S, Liu I, Otterness D, Dean NM, Abraham RT, Wang XF: Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. Genes Dev 2004;18:249–254.
21 Iijima K, Komatsu K, Matsuura S, Tauchi H: The Nijmegen breakage syndrome gene and its role in genome stability. Chromosoma 2004;113:53–61.
22 Lukas C, Falck J, Bartkova J, Bartek J, Lukas J: Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage. Nat Cell Biol 2003;5:255–260.
23 Lee JH, Paull TT: ATM Activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science 2005;308:551–554.
24 Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A: H2AX: the histone guardian of the genome. DNA Repair (Amst) 2004;3:959–967.
25 Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD: 53BP1, an activator of ATM in response to DNA damage. DNA Repair (Amst) 2004;3:945–952.
26 Stucki M, Jackson SP: MDC1/NFBD1: a key regulator of the DNA damage response in higher eukaryotes. DNA Repair (Amst) 2004;3:953–957.
27 Peng A, Chen PL: NFBD1/Mdc1 mediates ATR-dependent DNA damage response. Cancer Res 2005;65:1158–1163.
28 Goldberg M, Stucki M, Falck J, D'Amours D, Rahman D, Pappin D, Bartek J, Jackson SP: MDC1 is required for the intra-S-phase DNA damage checkpoint. Nature 2003;421:952–956.
29 Bachrati CZ, Hickson ID: RecQ helicases: suppressors of tumorigenesis and premature aging. Biochem J 2003;374:577–606.
30 Venkitaraman AR: DUBing down a tumour suppressor. Nat Cell Biol 2005;7:332–333.
31 Pichierri P, Franchitto A, Rosselli F: BLM and the FANC proteins collaborate in a common pathway in response to stalled replication forks. EMBO J 2004;23:3154–3163.
32 Nijman SM, Huang TT, Dirac AM, Brummelkamp TR, Kerkhoven RM, D'Andrea AD, Bernards R: The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol Cell 2005;17:331–339.

33 Parrilla-Castellar ER, Arlander SJ, Karnitz L: Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. DNA Repair (Amst) 2004;3:1009–1014.
34 Dang T, Bao S, Wang XF: Human Rad9 is required for the activation of S-phase checkpoint and the maintenance of chromosomal stability. Genes Cells 2005;10:287–295.
35 Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB: Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. Genes Dev 2004;18:1423–1438.
36 Wakeman TP, Kim WJ, Callens S, Chiu A, Brown KD, Xu B: The ATM-SMC1 pathway is essential for activation of the chromium[VI]-induced S-phase checkpoint. Mutat Res 2004;554:241–251.
37 Alderton GK, Joenje H, Varon R, Borglum AD, Jeggo PA, O'Driscoll M: Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway. Hum Mol Genet 2004;13:3127–3138.
38 Traven A, Heierhorst J: SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins. Bioessays 2005;27:397–407.
39 Ball HL, Myers JS, Cortez D: ATRIP binding to RPA-ssDNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation. Mol Biol Cell 2005;16:2372–2381.
40 Unsal-Kacmaz K, Sancar A: Quaternary structure of ATR and effects of ATRIP and replication protein A on its DNA binding and kinase activities. Mol Cell Biol 2004;24:1292–1300.
41 Furuya K, Poitelea M, Guo L, Caspari T, Carr AM: Chk1 activation requires Rad9 S/TQ-site phosphorylation to promote association with C-terminal BRCT domains of Rad4TOPBP1. Genes Dev 2004;18:1154–1164.
42 Musio A, Montagna C, Mariani T, Tilenni M, Focarelli ML, Brait L, Indino E, Benedetti PA, Chessa L, Albertini A, Ried T, Vezzoni P: SMC1 involvement in fragile site expression. Hum Mol Genet 2005;14:525–533.
43 Cortez D, Glick G, Elledge SJ: Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. Proc Natl Acad Sci USA 2004;101:10078–10083.
44 Stiff T, Reis C, Alderton GK, Woodbine L, O'Driscoll M, Jeggo PA: Nbs1 is required for ATR-dependent phosphorylation events. EMBO J 2005;24:199–208.
45 Park BJ, Kang JW, Lee SW, Choi SJ, Shin YK, Ahn YH, Choi YH, Choi D, Lee KS, Kim S: The haploinsufficient tumor suppressor p18 upregulates p53 via interactions with ATM/ATR. Cell 2005;120:209–221.
46 Lee J, Kumagai A, Dunphy WG: Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17. Mol Cell 2003;11:329–340.
47 Chini CC, Chen J: Claspin, a regulator of Chk1 in DNA replication stress pathway. DNA Repair (Amst) 2004;3:1033–1037.
48 Kumagai A, Kim SM, Dunphy WG: Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1. J Biol Chem 2004;279:49599–49608.
49 Zhao H, Russell P: DNA binding domain in the replication checkpoint protein Mrc1 of *Schizosaccharomyces pombe*. J Biol Chem 2004;279:53023–53027.
50 Sar F, Lindsey-Boltz LA, Subramanian D, Croteau DL, Hutsell SQ, Griffith JD, Sancar A: Human claspin is a ring-shaped DNA-binding protein with high affinity to branched DNA structures. J Biol Chem 2004;279:39289–39295.
51 Clarke CA, Clarke PR: DNA-dependent phosphorylation of Chk1 and Claspin in a human cell-free system. Biochem J 2005;388(Pt2):705–712.
52 Yoo HY, Kumagai A, Shevchenko A, Shevchenko A, Dunphy WG: Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. Cell 2004;117:575–588.
53 Lin SY, Li K, Stewart GS, Elledge SJ: Human Claspin works with BRCA1 to both positively and negatively regulate cell proliferation. Proc Natl Acad Sci USA 2004;101:6484–6489.
54 Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND: Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. EMBO J 2005;24:1157–1169.
55 Han EK, Butler C, Zhang H, Rosenberg S, Giranda VL, Ng SC, Luo Y: Chkl binds and phosphorylates BAD protein. Anticancer Res 2004;24:3907–3910.
56 Unsal-Kacmaz K, Mullen TE, Kaufmann WK, Sancar A: Coupling of human circadian and cell cycles by the timeless protein. Mol Cell Biol 2005;25:3109–3116.
57 King FW, Skeen J, Hay N, Shtivelman E: Inhibition of Chk1 by activated PKB/Akt. Cell Cycle 2004;3:634–637.

58 Puc J, Keniry M, Li HS, Pandita TK, Choudhury AD, Memeo L, Mansukhani M, Murty VV, Gaciong Z, Meek SE, Piwnica-Worms H, Hibshoosh H, Parsons R: Lack of PTEN sequesters CHK1 and initiates genetic instability. Cancer Cell 2005;7:193–204.
59 Krause DR, Jonnalagadda JC, Gatei MH, Sillje HH, Zhou BB, Nigg EA, Khanna K: Suppression of Tousled-like kinase activity after DNA damage or replication block requires ATM, NBS1 and Chk1. Oncogene 2003;22:5927–5937.
60 Groth A, Lukas J, Nigg EA, Sillje HH, Wernstedt C, Bartek J, Hansen K: Human Tousled like kinases are targeted by an ATM- and Chk1-dependent DNA damage checkpoint. EMBO J 2003;22:1676–1687.
61 Carrera P, Moshkin YM, Gronke S, Sillje HH, Nigg EA, Jackle H, Karch F: Tousled-like kinase functions with the chromatin assembly pathway regulating nuclear divisions. Genes Dev 2003;17:2578–2590.
62 Dozier C, Bonyadi M, Baricault L, Tonasso L, Darbon JM: Regulation of Chk2 phosphorylation by interaction with protein phosphatase 2A via its B′ regulatory subunit. Biol Cell 2004;96:509–517.
63 Ching RW, Dellaire G, Eskiw CH, Bazett-Jones DP: PML bodies: a meeting place for genomic loci? J Cell Sci 2005;118:847–854.
64 Dellaire G, Bazett-Jones DP: PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. Bioessays 2004;26:963–977.
65 Xu ZX, Zou WX, Lin P, Chang KS: A role for PML3 in centrosome duplication and genome stability. Mol Cell 2005;17:721–732.
66 Bracken AP, Ciro M, Cocito A, Helin K: E2F target genes: unraveling the biology. Trends Biochem Sci 2004;29:409–417.
67 Rogoff HA, Kowalik TF: Life, death and E2F: linking proliferation control and DNA damage signaling via E2F1. Cell Cycle 2004;3:845–846.
68 Stevens C, La Thangue NB: The emerging role of E2F-1 in the DNA damage response and checkpoint control. DNA Repair (Amst) 2004;3:1071–1079.
69 Hershko T, Chaussepied M, Oren M, Ginsberg D: Novel link between E2F and p53: proapoptotic cofactors of p53 are transcriptionally upregulated by E2F. Cell Death Differ 2005;12:377–383.
70 Urist M, Tanaka T, Poyurovsky MV, Prives C: p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. Genes Dev 2004;18:3041–3054.
71 Wang B, Liu K, Lin FT, Lin WC: A role for 14-3-3 tau in E2F1 stabilization and DNA damage-induced apoptosis. J Biol Chem 2004;279:54140–54152.
72 Winkles JA, Alberts GF: Differential regulation of polo-like kinase 1, 2, 3, and 4 gene expression in mammalian cells and tissues. Oncogene 2005;24:260–266.
73 Tsvetkov L, Stern DF: Phosphorylation of Plk1 at S137 and T210 is inhibited in response to DNA damage. Cell Cycle 2005;4:166–171.
74 van Vugt MA, Medema RH: Checkpoint adaptation and recovery: back with Polo after the break. Cell Cycle 2004;3:1383–1386.
75 Fisk HA, Mattison CP, Winey M: A field guide to the Mps1 family of protein kinases. Cell Cycle 2004;3:439–442.
76 Wei JH, Chou YF, Ou YH, Yeh YH, Tyan SW, Sun TP, Shen CY, Shieh SY: TTK/hMps1 participates in the regulation of DNA damage checkpoint response by phosphorylating CHK2 on threonine 68. J Biol Chem 2005;280:7748–7757.
77 Kramer A, Lukas J, Bartek J: Checking out the centrosome. Cell Cycle 2004;3:1390–1393.
78 Toji S, Yabuta N, Hosomi T, Nishihara S, Kobayashi T, Suzuki S, Tamai K, Nojima H: The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. Genes Cells 2004;9:383–397.

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The Role of the APC Tumor Suppressor in Chromosomal Instability

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Abstract

Colorectal cancer (CRC) still represents the model of choice to study the mechanisms underlying tumor initiation and progression. Accordingly, CRC has been central in the analysis of the role played by chromosomal instability (CIN) in tumor initiation and progression. Although loss of APC tumor suppressor function initiates the adenoma-carcinoma sequence in the vast majority of CRCs through constitutive activation of Wnt/β-catenin signaling, the *APC* gene also represents a candidate CIN gene in CRC. Accordingly, two studies published in 2001 showed that truncating *Apc* mutations can lead to both quantitative and qualitative ploidy changes in primary mouse cell lines, mainly due to kinetochore and centrosome abnormalities. Here, we review and discuss the more recent literature on APC's functional activities possibly related to its role in eliciting CIN in tumor initiation and progression. We propose a model where loss and/or truncation of APC cause mitotic spindle defects that, upon somatic inactivation of other putative CIN genes (e.g. spindle and cell cycle checkpoint genes, DNA repair, telomere maintenance, etc.) underlie aneuploidy as observed in the majority of CRCs.

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Chromosomal Instability in Tumorigenesis

Genetic instability has long been postulated as an essential condition for tumors to develop and progress towards more malignant stages. Chromosome number variations and loss of genome integrity in cancer have been observed since the very first cytological and molecular analyses, thus implying that cancer cells contain multiple gene mutations [1, 2]. Nevertheless, the majority of chromosomal abnormalities are not tumor-specific, which may indicate that genetic instability is an intrinsic feature of cancer cells [3]. The concept according to

which tumors develop through the accumulation of genetic alterations in oncogenes and tumor suppressor genes is widely accepted [4]. However, normal mutation rates are likely to be too low to allow the multiplicity of mutations observed in cancer cells. Hence, defects that increase mutation rates are essential to account for the large numbers of abnormalities observed in human tumors [3, 5, 6]. From this perspective, genetic instability, here referred to as an increased dynamic rate of changes, is likely to represent an essential prerequisite to accumulate the large number of alterations that occur during the tumorigenic process. The latter is supported by different mathematical models [7, 8]. However, alternative models indicate that the clonal evolution model is still in agreement with the concept of selection being the main driving force behind tumor initiation and progression [9, 10]. Colorectal cancer (CRC) has been instrumental in this debate as its different histological stages allow the dissection of the genetic events underlying tumor initiation and progression [11].

Chromosomal Instability in Colorectal Cancer

Colorectal tumor initiation and progression towards malignancy occur through well-defined histopathological and molecular steps, the so-called adenoma-carcinoma sequence [12]. Two main types of genetic instability have been recognized in human CRC, microsatellite instability (MSI or MIN) and chromosomal instability (CIN) [3]. MSI results from loss of mismatch repair (MMR) function and is earmarked by tumor-specific frame-shift mutations in stretches of short repetitive DNA sequences (microsatellite repeats) distributed throughout the genome [13, 14]. Notably, MIN tumors have increased nucleotide mutation rates when compared with normal cells but share near-diploid chromosomal contents [15–17]. Germline defects in MMR genes account for the hereditary non-polyposis colorectal cancer syndrome (HNPCC), an autosomal dominant predisposition to colorectal, uro-genital and skin cancers [18]. MSI and/or somatic defects in the same MMR genes are found in approximately 15% of sporadic colon cancers.

The first indication of the presence of specific genetic alterations underlying changes in tumor histology came from cytogenetics. Karyotype analyses of colorectal cancers have revealed characteristic patterns of chromosomal abnormalities [19–21]. In fact, the vast majority of CRCs are characterized by abnormal chromosomal contents with a heterogeneous and broad spectrum of both numerical and structural changes such as inversions, deletions, duplications and translocations [22]. These abnormalities define aneuploidy. Experimental evidence indicates that aneuploidy arises in these cancers as a result of CIN, here defined as the accelerated rate of gains or losses of entire chromosomes or

part of them [22, 23]. Clones from various colorectal cancer cell lines, expanded through a given number of passages and then analyzed by fluorscent in situ hybridization (FISH) with centromeric DNA probes of each individual chromosome, indicated that, whereas MSI cell lines are mostly near-diploid, microsatellite stable (MSS) colorectal cancer cells showed an increased frequency of chromosomal gains and losses at each cell division. CIN may provide additional growth advantage to the cancer cell by accelerating the rate of loss of heterozygosity at tumor suppressor loci and/or by amplifying chromosomal regions encompassing oncogenes. CIN may also represent a mechanism by which the cancer cell can fine-tune its growth characteristics to meet changes in the environment, thus possibly underlying therapeutic failures.

Over 90% of all CRCs show chromosomal aberrations, some of which are recurrent and represent key chromosomal changes underlying colorectal cancer initiation and progression, e.g. loss of chromosome 18, gain of chromosome 7, and other structural rearrangements, e.g. at chromosomes 1 p and 17 p. However, conventional cytogenetic analysis is limited by its low-resolution. In more recent years, the introduction of techniques like comparative genomic hybridization (CGH) and FISH, has allowed the high-resolution detection of chromosomal aberrations throughout the progression from low-grade adenoma to carcinoma [24]. By means of array CGH, Hermsen and colleagues showed evidence for the presence of specific subsets of chromosomal gains and losses strongly associated with adenoma-carcinoma progression [25].

To assess more functional aspects of genomic instability, colorectal cancer is often the experimental tumor of choice as it offers a well-defined model of stepwise progression, tissue samples from different stages are available from gastroenterology and surgical units for molecular analysis, and because a large number of well-characterized cell lines have been established from several CIN and MIN tumors. Analysis of CRC-derived cell lines that do not harbor MMR defects showed high rates of chromosome gain and loss, with acquisition of chromosomal changes at rates 10–100 times faster than in MMR-deficient cells [22]. These experiments also indicated that MSI and CIN may be mutually exclusive pathways of genetic instability in colorectal cancer [3]. However, the existence of a subgroup of colorectal cancers with apparently stable, near-diploid chromosomes and stable microsatellites (MACS) was more recently reported [26].

To date, many potential mechanisms have been shown to play a role in CIN by contributing to aneuploidy: mitotic and cell cycle checkpoints, telomere shortening and telomerase expression, centrosome number regulation, double-strand break repair, kinetochore function, and chromatid segregation [3, 27] (table 1). In particular, mutations at the mitotic checkpoint genes *BUB1* and *BUBR1* [28, 29], the cyclin E regulator *CDC4* [30], and the *TP53* tumor suppressor [31] have been implicated as rate-limiting events in eliciting CIN in CRC. However,

Table 1. Possible mechanisms of chromosomal instability in tumors and a selection of genes involved

Cell structural defect	Mechanisms leading to CIN	Genes implicated	References
Centrosome	Segregation defect	p53 pathway, *ATR*, *BRCA1*, *BRCA2*, *XRCC2/3*, *RAD6*, Aurora-A, Survivin	31; 125–133
Mitosis check point	Chromosome missegregation	*BUB1/BUBR1*, *MAD2*	28; 29; 134; 135
DNA damage check point	Enhanced/aberrant mitotic recombination	*ATM*, *ATR*, *BML*, *BRCA2*, *FANCA-L*, *NBS1*	136–142
Microtubules and spindle dynamics	Spindle defects	*APC*, *GSK3*-β	35; 36; 69; 97; 98; 143; 144
Kinetochore assembly	Premature anaphase	*HEC1*	145
Chromatin cohesion and chromosome condensation	Disjunction failure	Securin/*PTTG*	146; 147
Cell cycle control	Cell cycle disturbance	*TP53*, *CDC4*, *CCNE1*	30; 148–150
Telomeres	Chromosome fusion	*PINX1*, *TERC*, *TERF1/PIN2*	151
Cytokinesis	Multinucleate cells	*BRACA2*	123

mutations in these alleged CIN genes are relatively rare [28, 29], and occur at late stages of the adenoma-carcinoma sequence [4], whereas aneuploid changes have been observed already at early adenoma stages [32–34]. More recently, we and others have found that mutations in *APC* give rise to polyploid and aneuploid chromosomal changes in mouse primary cell lines and may therefore trigger CIN at the very start of the adenoma-carcinoma sequence [35, 36]. Here, we will review the functional aspects of the APC tumor suppressor protein with respect to chromosomal stability at mitosis and its role in CIN in CRC.

Functional Aspects of the APC Tumor Suppressor Protein

Inactivation of the adenomatous polyposis coli (*APC*) tumor suppressor gene represents the very first and rate-limiting step of the adenoma-carcinoma sequence in CRC. Notwithstanding its well-known multi-functionality [37], APC's main tumor suppressing function resides in its capacity to regulate Wnt signaling as elegantly shown by the identification of β-catenin (*CTNNB1*) gene mutations in sporadic colorectal cancers with an intact *APC* gene [38, 39]. β-catenin is the main intracellular signaling protein of the canonical Wnt pathway and its cellular level is regulated by a 'destruction' complex composed by several proteins among which there is APC (see below). Loss of APC function or oncogenic β-catenin mutations that make it resistant to APC-driven proteolytic degradation, result in the constitutive activation of the Wnt signal transduction pathway that regulates epithelial homeostasis along the intestinal villus-crypt axis [11, 40]. Notwithstanding the latter, the *APC* gene encodes a 312 kDa protein encompassing multiple and diverse functional motifs that, upon APC truncation and/or complete loss of function, may play additional roles in tumor progression and malignant transformation [37] (fig. 1).

The N-terminus of APC contains several regions of heptad repeats responsible for the formation of coiled-coil domains and often involved in oligodimerization [41–43]. Whereas the N-terminal 55 amino acids of APC form a dimeric coiled-coil [41], residues 129–250 can result in an intramolecular coiled-coil [44]. The N-terminus of APC also encompasses two nuclear export sequences (NES), both required for shuttling APC between nucleus and cytoplasm [45, 46]. Seven armadillo (ARM) repeats, a motif first found in the fruit fly β-catenin homolog Armadillo, are also found in the N-terminal region of APC [47]. Additional binding motifs for the protein phosphatase PP2A, the guanine nucleotide exchange factor (GEF) Asef, and Kap3, a linker protein for kinesins [48–50], partially overlap with the ARM repeats. Although it is not clear how these complex interactions occur and are regulated at specific phases of the cell

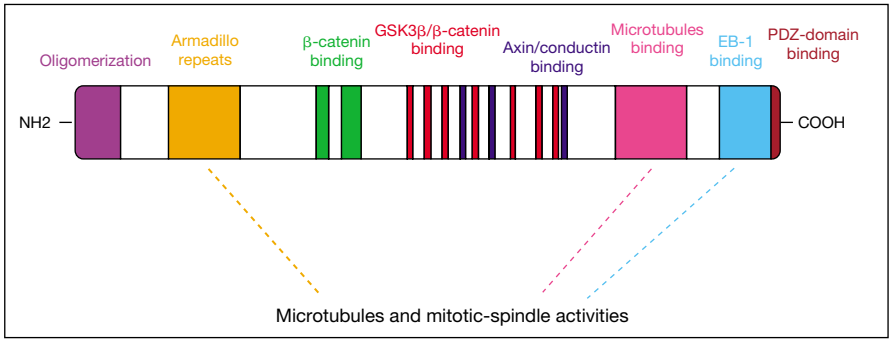

Fig. 1. Schematic representation of the APC protein with its functional motifs.

cycle and in specific cellular types, it is important to point out that truncated APC proteins encompassing the N-terminus positively affect GEF activity and cell motility by Asef when compared with full length APC [51]. The latter is consistent with the alleged dominant-negative effect exerted by truncating *APC* mutations on apoptosis, cell motility and cytoskeletal organization [52, 53]. The recent report of the putative interaction between the N-terminal third of APC and its C-terminal region may support this hypothesis [54].

The middle region of the APC protein encompasses the domains responsible for β-catenin regulation in Wnt signaling. Three 15 a.a. and seven 20 a.a. repeats mediate binding and downregulation of β-catenin, respectively. Three Ser-Ala-Met-Pro repeats (SAMP) are interspersed among the 20 amino acid repeats and allow interaction with the scaffold proteins axin/conductin [47].

APC regulates Wnt signaling by catalyzing the formation of a multiprotein complex, the so-called 'destruction complex', comprehensive of the scaffolding proteins axin and conductin, the glycogen synthase kinase 3β (GSK3β) and casein kinase (CKI), and APC itself [55–58]. In the absence of the Wnt ligand, the destruction complex is formed which results in Ser/Thr-phosphorylation of β-catenin and its subsequent proteosomal degradation [59–61]. In the presence of the Wnt ligand, Dishevelled (Dsh) inactivates GSK3β, thus resulting in the intracellular stabilization of β-catenin and its nuclear translocation. Once in the nucleus, β-catenin binds to DNA-binding proteins of the T-cell factor (TCF) family, to serve as an essential co-activator of transcription [62, 63]. The activity of TCF is tightly controlled, as TCFs are complexed with potent co-repressors such as Groucho in the absence of Wnt signaling [64].

Finally, the C-terminal third of the APC protein, the least conserved throughout evolution, encompasses distinct domains that mediate interactions with several cytoskeletal proteins. A stretch of approximately 200 amino acids, enriched

in positive charges, is responsible for the interaction of APC with microtubules (MT) [65–67]. The C-terminal 170 amino acids of APC bind the end binding protein 1 (EB1), a small microtubule end-binding protein [68], whereas the very last 15 residues encompass the binding site for PDZ domains [69, 70].

Although previous reports indicated active nuclear-cytoplasmic shuttling of APC [71] and its putative role in transcriptional regulation via its ability to bind DNA [72], to date there is no evidence that APC directly acts as a transcriptional regulator. Moreover, a recent study questioned the specificity of many commonly used anti-APC antibodies and showed that both wild-type and truncated APC were primarily cytoplasmic in colon cancer cells, but increased in the nucleus after leptomycin B treatment, consistent with CRM1-dependent nuclear export [73]. The predominantly cytoplasmic subcellular localization of APC possibly reflects its physical interaction with the cytoskeleton. APC can bind both directly and indirectly to microtubules [66, 67], and clusters at the distal tips (the 'plus ends') of microtubules in cellular protrusion of migrating cells [74]. The association with the plasma membrane is highly dynamic and requires an intact actin cytoskeleton [75]. In highly polarized cells from the inner ear, APC localizes at the plus end of microtubules oriented towards the basal membrane [76]. The observation that truncated APC proteins lacking the MT binding site fail to interact with the microtubules though retaining KAP3 binding, suggests that the relation of APC with the cytoskeleton is due to a combination of direct and indirect interactions that possibly modulate its subcellular distribution [50]. APC has been shown to promote microtubule polymerization in vitro [66, 77]. Also, microtubules bound to APC are more stable under depolymerizing conditions both in vivo and in vitro [78]. Notably, Cdc42-dependent phosphorylation of GSK3β occurs specifically at the leading edge of migrating cells, and induces the interaction of APC with the plus ends of microtubules, essential to promote cell polarization and control the direction of cell protrusion [79]. The latter suggests that interaction of APC with the cytoskeleton, similar to its interaction with β-catenin, is regulated by binding to GSK3β. Accordingly, binding of APC to microtubules is modulated by phosphorylation [78], suggesting that APC is alternatively employed as scaffolding protein in the regulation of Wnt/β-catenin signaling and as microtubule-stabilizing protein in the regulation of cell polarity and migration.

Truncated APC proteins lacking the N-terminal ARM repeats or the C-terminal MT-binding site fail to form proper aggregates at the plus ends of the microtubules, thus affecting their subcellular distribution pattern [80].

An additional interaction between microtubules and APC occurs through EB1. EB1, initially isolated by a two-hybrid screen as a protein binding to the C-terminus of APC [81], is highly conserved throughout evolution, from yeast to mammals. EB1-like proteins have been shown to be involved in almost all the

microtubule-based processes including maintenance of cell polarity, anchorage to nucleation sites, and the regulation of spindle formation and chromosome segregation at mitosis [82, 83]. The C-terminal domain of the EB1 protein interacts with the C-terminus of APC [84–86], and this APC/EB1 complex has been described to stabilize microtubule ends in vivo [87]. However, in colon cancer cell lines carrying truncated APC not encompassing the EB1-binding site, endogenous EB1 localization is unchanged, suggesting that EB1 can bind to microtubule tips independently of APC [88, 89]. Also, CRC cell lines transfected with several different GFP-mutant APC show that the interaction with EB1 is responsible for directing APC to the tip of microtubules. In fact only the mutant APC that express the EB1 binding motif show localization at the plus ends of microtubules [68].

A more recent study [90] suggests that the major EB1 interaction site in APC involves the dipeptide segment 2805–2806 and that the same interaction is negatively regulated by cyclin-dependent mitotic kinase Cdc2 phosphorylation of the Ser2789 residue of APC. Thus, C-terminal phosphorylation of APC appears to play a key role in the regulation of its interaction with EB1 throughout the cell cycle and in particular during mitosis [68].

Although the majority of *APC* mutations in human tumors encode truncated proteins lacking the EB1 binding domain [91, 92], no cancer-associated mutations in the *EB1* gene have been found to date [93]. Moreover, mice carrying targeted *Apc* truncations that remove the EB1-binding motif without affecting its capacity to regulate Wnt/β-catenin signaling, do not develop tumors [94]. However, the possibility remains that loss of EB1-APC binding plays a role in tumor progression by affecting cell migration, polarity and/or chromosomal segregation (see below).

A Role for APC in Chromosomal Instability

During mitosis, APC clusters at the plus-ends of the spindle microtubules and co-localizes with the kinetochore, the attachment site of the mitotic spindle to the newly duplicated chromosomes [35, 36] (fig. 2). These observations strongly suggest a role for APC in mitotic spindle formation and chromosome segregation. Multicolor FISH analysis of mouse embryonic stem (ES) cell lines homozygous for the *Min* allele, lacking the C-terminal third of the protein, revealed ploidy defects and structural chromosomal aberrations. Genetic evidence that the observed chromosomal abnormalities do not result from Wnt signaling defects but from deletion of the C-terminal APC functional domains was provided by the confirmation of the aneuploid and polyploid changes in ES lines homozygous for the *Apc*1638T targeted mutation, previously shown to retain wild type β-catenin

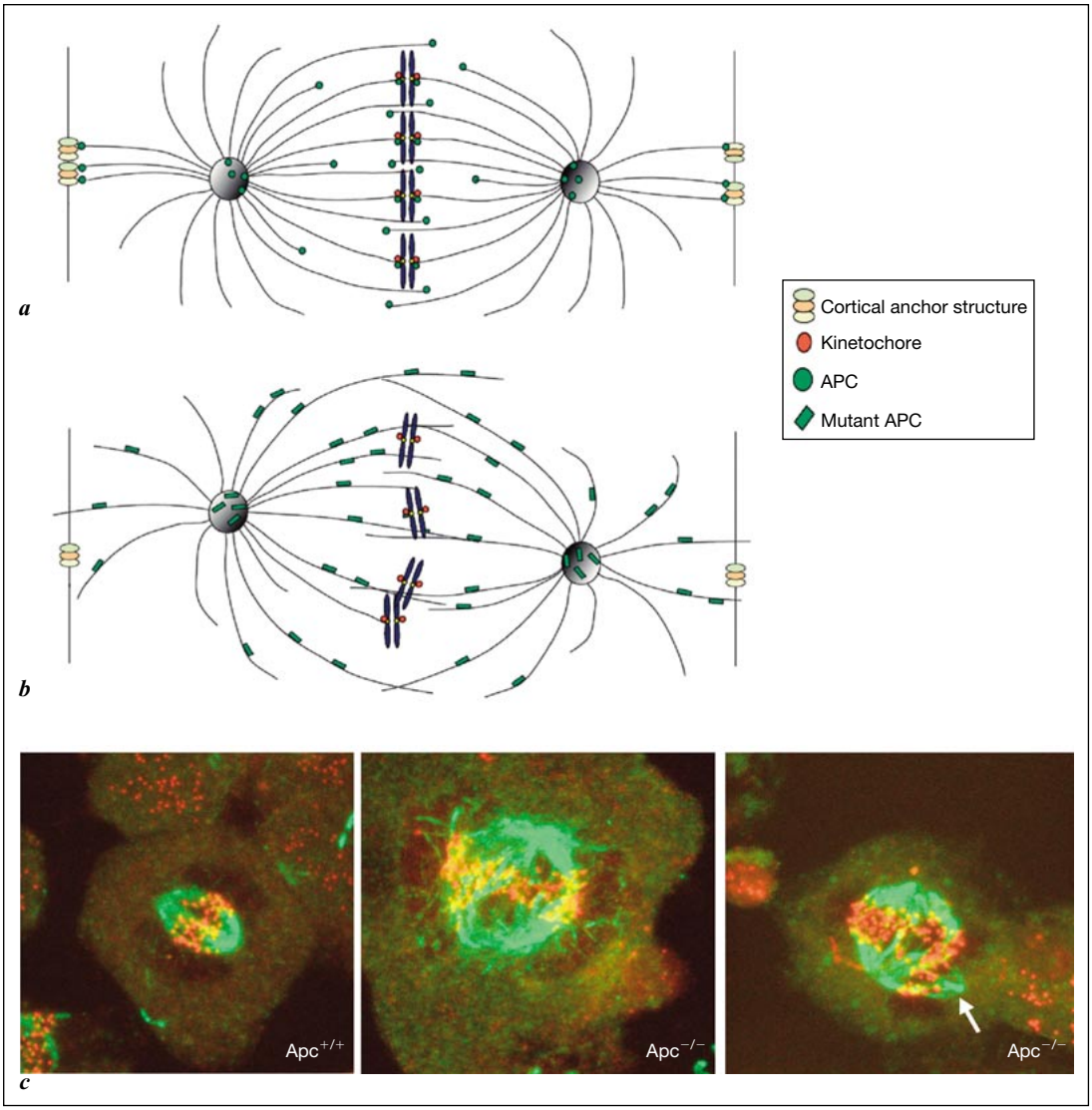

Fig. 2. Mitotic spindle defects in *Apc* mutant cells. In (***a***) a representation of a normal mitotic figure with localization of APC at the kinetochore and at the plus-ends of the microtubules is shown. The hypothetical binding of APC on astral microtubules to the cortical anchor structure allows to keep the mitotic spindle parallel to the epithelial plane. In (***b***) there is represented a mitosis in an APC mutant cell with defects in the spindle organization and the failure of the APC binding to the kinetochore and to the cortical anchor structure. In (***c***) examples of spindles formed in *Apc* wild type and mutant ES cells lines are shown. In green the β-tubulin staining marks the spindle, whereas in red the CREST/kinetochore structure is stained. Note in the *Apc* mutants the spindle abnormalities with most of the microtubules projecting in a chaotic manner in the cytoplasm. In the right $Apc^{-/-}$ picture, the white arrow designates an extra centrosome.

regulatory function [35, 94]. The co-localization of APC at the kinetochore of the metaphase chromosomes is abolished by colcemid treatment, an agent that selectively depolymerizes microtubules. Microtubule staining of *Apc* mutant ES cells shows a disorganized mitotic spindle with most microtubules projecting randomly into the cytoplasm in contrast with the properly aligned spindles observed in wild-type cells (fig. 2). Also, *Apc*-mutant ES lines showed high incidence of mono- and multicentric spindles and supernumerary centrosomes at mitosis when compared with wild type ES cells [35, 36]. Notably, APC and EB1 co-localize with the centrosome in both mammalian and *Xenopus* mitotic cells, and are thus likely to play a role in centrosomally driven spindle formation by anchoring cytoplasmic MT minus ends to the centriole [35, 36, 69, 95, 96]. However, the latter could also represent a consequence of APC's role in directly supporting spindle formation and stability in vivo, as suggested by the observation of microtubule-resistance against nocodazole treatment in cells expressing full-length or C-terminal APC [78]. Moreover, APC acts, possibly within a protein complex together with EB1 and the formin mDia, a component of the Rho-GTPase pathway, to selectively stabilize microtubules in fibroblasts [87].

Another observation of APC involvement in CIN comes from an experiment in which the overexpression of a dominant-negative C-terminal APC fragment encompassing the EB1-binding domain in the APC-proficient and near-diploid HTC116 CRC cell line resulted in a 2.5–5 fold increase in the frequency of numerical chromosomal aberrations [97].

Since these first reports, additional studies have provided additional insights on the role of loss and/or truncation of APC in eliciting CIN. Green and Kaplan [98] have shown that CIN tumor cells exhibit inefficient microtubule plus-end attachments during mitosis, accompanied by impairment of chromosome alignment at metaphase. These abnormalities correlate with the *APC* mutational status. Notably, it was also found that a single truncating mutation in APC acts dominantly to interfere with microtubule plus-end attachments and causes a dramatic increase in mitotic abnormalities [98]. Consistent with the latter, N-terminal APC fragments expressed in HCT-116, a near diploid colon cancer cell line with two wild-type *APC* alleles, result in spindle checkpoint defect and aneuploidy [97]. Although these alleged dominant-negative effects of truncated APC need to be confirmed by additional in vitro and in vivo studies with knock-in and inducible *APC* mutations to exclude artifacts due to changes in the expression levels and subcellular localization of the transfected recombinant proteins, they may have important implications for the further elucidation of APC-driven tumorigenesis. In a previous study by somatic cell fusion analysis, MSI was shown to behave as a recessive trait, whereas CIN appeared to be dominant [22]. However, complete loss of APC function also results in spindle abnormalities similar to those observed in cells with truncated APC: depletion

of APC from *Xenopus* extracts leads to a decrease in microtubule density and changes in tubulin distribution in spindles and asters [69].

Notably, it has also been observed that the level of Bub1p was enriched at kinetochores during metaphase in cells transfected with the truncated APC construct [98]. Similarly, kinetochore-bound BubR1 is also significantly increased in HTC-116 expressing a mutant N-terminal APC fragment [97]. This is of interest in view of a previous study showing that the dissociation of Bub1 and BubR1 from the kinetochores of aligned chromosomes depends on microtubule attachment and tension, respectively [99]. The observed stabilization of checkpoint proteins at the kinetochore during metaphase may indeed result from the dominant-negative effects exerted by truncated APC on kinetochore-microtubule attachment and tension.

In mitotic cells, APC and Bub3 are juxtaposed in prometaphase and metaphase along kinetochore microtubules, as also confirmed by co-immunoprecipitation [36]. These observations, together with the fact that Bub1-Bub3 and BubR1-Bub3 complexes can phosphorylate APC in vitro with high specificity, are suggestive of a role for APC phosphorylation by Bub kinases in the regulation of kinetochore-microtubule attachment through the different stages of mitosis.

An additional function of APC in spindle formation and stabilization possibly related to its capacity to elicit CIN when mutated, lies in its microtubule-capture activity at the cell-cortex, homologous to the yeast cortical microtubule capture site composed by Bim1 (homolog of mammalian EB1) and Kar9 [100, 101]. In yeast, Kar9 represents a central link between the actin cytoskeleton and microtubules in establishing correct spindle orientation [102, 103]. Although Kar9 does not seem to be conserved throughout evolution, functional similarities can be observed between Kar9 and the C-terminus of APC encompassing the EB1-binding site [104]. Recently, APC has been described to be distributed in a punctuated fashion along the path of microtubule growth and, in addition, to localize at the basal cortex of mammalian cells where it provides attachment for growing microtubules thus contributing to the organization and stabilization of the microtubule network at the cortex [101]. Interestingly, a direct APC-EB1 interaction is not required for either APC or EB1 localization to the microtubules at the basal cortex [101].

Hence, APC, like Kar9, is a component of the cortical template and may act in mammalian cells as a guide to capture microtubules at specific sites along the plasma membrane. Whether this anchor function also ensures the correct position of the mitotic spindle and thus chromosomal segregation during mitosis is a fascinating though yet to be demonstrated hypothesis.

In summary, the above data indicate that, during mitosis, APC contributes both to spindle formation radiating from the centrosome, and to the proper

attachment of the growing ends of microtubules to the kinetochore (either directly and/or through EB1). Hence, *APC* mutations are likely to affect several structural complexes and pathways (kinetochore, microtubules, centrosomes, and spindle checkpoints) known to be implicated in both numerical and structural CIN [105] (fig. 2). Accordingly, both tetraploidy and aneuploidy were found to represent the main chromosomal defects in mouse primary *Apc*-mutant cell lines [35, 36].

In vivo Consequences of *APC* Mutations on Chromosomal Instability

Although loss of APC-mediated regulation of Wnt/β-catenin signaling represents the main initiating event in colorectal tumorigenesis, it is safe to assume that *APC* mutation affects a multitude of additional cellular functions thus providing the nascent tumor with additional selective advantages likely to play important roles in progression towards malignancy. The latter is also suggested by the observation according to which tumors with oncogenic β-catenin mutations are usually smaller and less aggressive than those with *APC* mutations [106]. Accordingly, additional defects in cell migration, apoptosis, and differentiation have been reported in mouse cells carrying *Apc* truncating mutations [53, 107, 108].

Whether loss of APC function directly results in CIN is at present still a matter of debate. In general, aneuploidy has been reported in larger adenomas, suggesting its increase with tumor progression [25, 32]. Shih and colleagues [34] demonstrated allelic imbalance (AI), indicative of losses or gains of defined chromosomal regions, in small benign colorectal tumors thus suggesting that CIN occurs at very early stages during colorectal neoplasia. On the other hand, Sieber et al. [109] failed to detect any chromosomal changes in the majority of *APC*-mutant adenomas analyzed. These apparent discrepancies may be explained by the different detection sensitivity of the methods employed in the two studies, namely digital PCR vs. a combination of flow cytometry and conventional CGH and LOH analyses [34, 109]. In a more recent study, we have shown aneuploid changes at specific chromosomal regions (Cardoso et al., submitted) in a small but significant fraction of FAP adenomas with established *APC* mutations by array CGH. This approach allows to measure relative DNA abundances with a sensitivity that theoretically ranges from abnormalities affecting complete chromosome arms to few megabases. Digital PCR, when employed to assess AI, is even more sensitive than array CGH to detect gain and loss events affecting chromosomal regions <1 Mb. Both digital PCR and array CGH are also likely to detect ploidy changes present in subpopulations of

neoplastic cells that only become completely clonal at later stages of tumorigenesis. In contrast, although flow-cytometry is the method of choice when analyzing ploidy status of tumor samples, aneuploid changes below specific thresholds are likely to go undetected. Therefore, based on the above functional and observational studies, we hypothesize that loss of the MT- and EB1-binding functions of APC, triggers a broad spectrum of mitotic spindle defects that may elicit a subtle but significantly increased rate of CIN. As truncated APC proteins may act in a dominant-negative fashion in eliciting similar mitotic defects [53, 97, 98], CIN may even precede the rate-limiting somatic hit at the wild type *APC* allele.

Discussion

CIN in CRC has been previously associated with mitotic spindle checkpoint defects [28]. Cells with defective spindle checkpoint prematurely exit mitosis after colcemid treatment. However, these findings contrast with the report by Tighe and colleagues that CIN cells do undergo mitotic arrest in response to spindle damage and have a robust checkpoint [110]. *Apc*-mutant ES cells also fail to show differences in mitotic index in comparison to wild type line [35], whereas analysis of *Mad2*- or *Bub3*-mutant embryonic cells revealed a dramatic drop in mitotic index levels [111, 112], due to the mitotic arrest upon microtubule depolymerization. Mutations in known spindle checkpoint genes are found in human cancers, though at overall low frequencies [27–29, 113].

It seems plausible that loss of APC function is necessary but insufficient to result in full-blow CIN. In *APC*-mutant aberrant crypt foci and small adenomas, a subtle but significant defect in microtubule dynamics and mitotic spindle assembly may occasionally lead to ploidy changes. Additional mutations in different categories of genes involved in mitotic and cell cycle checkpoints, telomere shortening and telomerase expression, centrosome number regulation, and double-strand break repair [3, 27] may work synergistically with the kinetochore and chromosome segregation defects caused by *APC* mutation in eliciting CIN. In fact, CIN may initially be suppressed by active cell cycle and mitotic checkpoints. Experimental evidence for the latter was provided by studies in $Apc^{Min/+}/BubR1^{+/-}$ compound mutant mice [114]. While $BubR1^{+/-}$ animals do not develop colonic tumors, compound $Apc^{Min/+}/BubR1^{+/-}$ mice are affected by increased intestinal tumor multiplicity and progression towards advanced stages when compared with $Apc^{Min/+}$ animals. Mouse embryonic fibroblasts (MEFs) derived from $Apc^{Min/+}/BubR1^{+/-}$ embryos show enhanced mitotic slippage in the presence of nocodazole and exhibit a higher rate of genomic instability than that of wild type or $BubR1^{+/-}$ or $Apc^{Min/+}$ MEFs, as indicated by

premature separation of sister chromatids, increased rates of micronuclei formation and aneuploid metaphases. It is therefore likely that even haploinsufficiency at spindle checkpoint genes like *BubR1* may elicit CIN in the presence of the mitotic spindle assembly defect caused by *APC* mutation.

Notwithstanding the above, the spectrum of genes that may act synergistically with APC in eliciting CIN in CRC is likely to extend beyond those implicated in checkpoint functions. Supporting evidence for the existence of specific CIN-genes able to synergize with APC to exert genomic instability has been provided by studies with *Apc*-mutant mouse models. Expression of *CDX2*, a homeodomain transcriptional factor involved in the development, differentiation and physiology of the intestinal epithelial lining, is markedly reduced in the later stages of human colorectal carcinogenesis, namely, high grade dysplasia and invasive carcinoma [115, 116]. Compound heterozygous $Apc^{+/\Delta 716}/Cdx2^{+/-}$ mice show a marked increase in colonic polyp formation when compared with the single mutant animals, with lesions frequently characterized by an increased anaphase bridge index (ABI), indicative of CIN [117]. Notably, normal colonic epithelium from compound heterozygous animals shows an increased ABI when compared to single mutant littermates, indicative of a putative synergism between *APC* and *CDX2* mutations in eliciting CIN.

In general, telomere abnormalities are known to be associated with promotion of epithelial cancer [118, 119], and genes related to telomere maintenance may co-operate with *APC* to exert CIN in CRC. Indeed, progressive telomere dysfunction increases intestinal tumor multiplicity in the second and third generation $Apc^{Min/+}/Terc^{-/-}$ mice, when telomere shortening becomes critical [120]. Notably, these tumors are characterized by high ABI incidence leading to the formation of dicentric chromosomes and high rate of chromosome loss. Also, the presence of short telomeres in colorectal carcinoma [121, 122], together with the increase of anaphase bridges at the adenoma-carcinoma transition [120] points to a role for defective telomere dynamics in genomic instability during colorectal carcinoma progression.

Recently, it has been reported that loss of the *BRCA2* tumor suppressor gene causes a cytokinesis defect, i.e. an impairment of the completion of cell separation upon mitosis [123]. As for *APC*, *BRCA2* was already known to cause both qualitative (aneuploidy) and quantitative (polyploidy) chromosomal changes. Whereas the first can be explained by the failure to repair double strand DNA breaks by mutant *BRCA2*, the latter appears now to result from impeded cell separation accompanied by abnormalities in myosin organization during late stages of cytokinesis. Accordingly, BRCA2 was shown to localize to the cytokinetic midbody [123]. Though no evidence to date has been reported for a role of APC in cytokinesis, tetraploidy is a main feature of *APC*-mutant cells [35, 36] and it may represent the primary consequence of the loss of its

multiple functional roles in mitosis (attachment of the mitotic spindle to the kinetochore, centrosome and cell cortex) and a first step towards aneuploidy. The subtlety of this initial and random CIN defect might represent the 'just-right' type of genetic instability [124] as it allows the occasional generation of cells carrying specific genetic defects on which clonal selection operates without excessive accumulation of genetic damage above viability thresholds. Additional somatic mutations in different categories of genes will act in a cumulative and/or synergistic fashion to progressively elicit increasing levels of CIN along the adenoma-carcinoma sequence.

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References

1 Boveri T: Zur Frage der Entstehung maligner Tumoren. Gustav Fisher Verlag, Jena, 1914.
2 Loeb LA, Springgate CF, Battula N: Errors in DNA replication as a basis of malignant changes. Cancer Res 1974;34:2311–2321.
3 Lengauer C, Kinzler KW, Vogelstein B: Genetic instabilities in human cancers. Nature 1998;396: 643–649.
4 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL: Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–532.
5 Loeb LA: Mutator phenotype may be required for multistage carcinogenesis. Cancer Res 1991;51: 3075–3079.
6 Loeb LA, Loeb KR, Anderson JP: Multiple mutations and cancer. Proc Natl Acad Sci USA 2003;100:776–781.
7 Michor F, Iwasa Y, Nowak MA: Dynamics of cancer progression. Nat Rev Cancer 2004;4:197–205.
8 Michor F, Iwasa Y, Vogelstein B, Lengauer C, Nowak MA: Can chromosomal instability initiate tumorigenesis? Semin Cancer Biol 2005;15:43–49.
9 Tomlinson IP, Novelli MR, Bodmer WF: The mutation rate and cancer. Proc Natl Acad Sci USA 1996;93:14800–14803.
10 Tomlinson I, Bodmer W: Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. Nat Med 1999;5:11–12.
11 Fodde R, Smits R, Clevers H: APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55–67.
12 Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990;61:759–767.
13 Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M: Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 1993;363: 558–561.
14 Thibodeau SN, Bren G, Schaid D: Microsatellite instability in cancer of the proximal colon [see comments]. Science 1993;260:816–819.

15 Parsons R, Li GM, Longley MJ, Fang WH, Papadopoulos N, Jen J, de la Chapelle A, Kinzler KW, Vogelstein B, Modrich P: Hypermutability and mismatch repair deficiency in RER+ tumor cells. Cell 1993;75:1227–1236.
16 Bhattacharyya NP, Skandalis A, Ganesh A, Groden J, Meuth M: Mutator phenotypes in human colorectal carcinoma cell lines. Proc Natl Acad Sci USA 1994;91:6319–6323.
17 Eshleman JR, Casey G, Kochera ME, Sedwick WD, Swinler SE, Veigl ML, Willson JK, Schwartz S, Markowitz SD: Chromosome number and structure both are markedly stable in RER colorectal cancers and are not destabilized by mutation of p53. Oncogene 1998;17:719–725.
18 Lynch HT, Lynch J: Lynch syndrome: genetics, natural history, genetic counseling, and prevention. J Clin Oncol 2000;18:19S–31S.
19 Mitelman F: Catalog of Chromosome Aberrations in Cancer. Wiley-Liss, New York, 1991.
20 Muleris M, Delattre O, Olschwang S, Dutrillaux AM, Remvikos Y, Salmon RJ, Thomas G, Dutrillaux B: Cytogenetic and molecular approaches of polyploidization in colorectal adenocarcinomas. Cancer Genet Cytogenet 1990;44:107–118.
21 Bardi G, Sukhikh T, Pandis N, Fenger C, Kronborg O, Heim S: Karyotypic characterization of colorectal adenocarcinomas. Genes Chromosomes Cancer 1995;12:97–109.
22 Lengauer C, Kinzler KW, Vogelstein B: Genetic instability in colorectal cancers. Nature 1997;386:623–627.
23 Thiagalingam S, Laken S, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B, Lengauer C: Mechanisms underlying losses of heterozygosity in human colorectal cancers. Proc Natl Acad Sci USA 2001;98:2698–2702.
24 Ried T, Knutzen R, Steinbeck R, Blegen H, Schrock E, Heselmeyer K, du Manoir S, Auer G: Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. Genes Chromosomes Cancer 1996;15:234–245.
25 Hermsen M, Postma C, Baak J, Weiss M, Rapallo A, Sciutto A, Roemen G, Arends JW, Williams R, Giaretti W, De Goeij A, Meijer G: Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. Gastroenterology 2002;123:1109–1119.
26 Chan TL, Curtis LC, Leung SY, Farrington SM, Ho JW, Chan AS, Lam PW, Tse CW, Dunlop MG, Wyllie AH, Yuen ST: Early-onset colorectal cancer with stable microsatellite DNA and near-diploid chromosomes. Oncogene 2001;20:4871–4876.
27 Wang Z, Cummins JM, Shen D, Cahill DP, Jallepalli PV, Wang TL, Parsons DW, Traverso G, Awad M, Silliman N, Ptak J, Szabo S, Willson JK, Markowitz SD, Goldberg ML, Karess R, Kinzler KW, Vogelstein B, Velculescu VE, Lengauer C: Three classes of genes mutated in colorectal cancers with chromosomal instability. Cancer Res 2004;64:2998–3001.
28 Cahill DP, Lengauer C, Yu J, Riggins GJ, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B: Mutations of mitotic checkpoint genes in human cancers. Nature 1998;392:300–303.
29 Shichiri M, Yoshinaga K, Hisatomi H, Sugihara K, Hirata Y: Genetic and epigenetic inactivation of mitotic checkpoint genes *hBUB1* and *hBUBR1* and their relationship to survival. Cancer Res 2002;62:13–17.
30 Rajagopalan H, Jallepalli PV, Rago C, Velculescu VE, Kinzler KW, Vogelstein B, Lengauer C: Inactivation of hCDC4 can cause chromosomal instability. Nature 2004;428:77–81.
31 Fukasawa K, Choi T, Kuriyama R, Rulong S, Vande Woude GF: Abnormal centrosome amplification in the absence of p53. Science 1996;271:1744–1747.
32 Giaretti W: A model of DNA aneuploidization and evolution in colorectal cancer. Lab Invest 1994;71:904–910.
33 Bardi G, Parada LA, Bomme L, Pandis N, Willen R, Johansson B, Jeppsson B, Beroukas K, Heim S, Mitelman F: Cytogenetic comparisons of synchronous carcinomas and polyps in patients with colorectal cancer. Br J Cancer 1997;76:765–769.
34 Shih IM, Zhou W, Goodman SN, Lengauer C, Kinzler KW, Vogelstein B: Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. Cancer Res 2001;61:818–822.
35 Fodde R, Kuipers J, Rosenberg C, Smits R, Kielman M, Gaspar C, van Es JH, Breukel C, Wiegant J, Giles RH, Clevers H: Mutations in the *APC* tumour suppressor gene cause chromosomal instability. Nat Cell Biol 2001;3:433–438.
36 Kaplan KB, Burds AA, Swedlow JR, Bekir SS, Sorger PK, Nathke IS: A role for the adenomatous polyposis coli protein in chromosome segregation. Nat Cell Biol 2001;3:429–432.

37 Fodde R: The multiple functions of tumour suppressors: it's all in APC. Nat Cell Biol 2003;5: 190–192.
38 Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 1997;275:1787–1790.
39 Sparks AB, Morin PJ, Vogelstein B, Kinzler KW: Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 1998;58:1130–1134.
40 Gaspar C, Fodde R: APC dosage effects in tumorigenesis and stem cell differentiation. Int J Dev Biol 2004;48:377–386.
41 Day CL, Alber T: Crystal structure of the amino-terminal coiled-coil domain of the APC tumor suppressor. J Mol Biol 2000;301:147–156.
42 Joslyn G, Richardson DS, White R, Alber T: Dimer formation by an N-terminal coiled coil in the APC protein. Proc Natl Acad Sci USA 1993;90:11109–11113.
43 Lupas A, Van Dyke M, Stock J: Predicting coiled coils from protein sequences. Science 1991;252:1162–1164.
44 Tickenbrock L, Cramer J, Vetter IR, Muller O: The coiled coil region (amino acids 129–250) of the tumor suppressor protein adenomatous polyposis coli (APC). Its structure and its interaction with chromosome maintenance region 1 (Crm-1). J Biol Chem 2002;277:32332–32338.
45 Henderson BR: Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat Cell Biol 2000;2:653–660.
46 Neufeld KL, Nix DA, Bogerd H, Kang Y, Beckerle MC, Cullen BR, White RL: Adenomatous polyposis coli protein contains two nuclear export signals and shuttles between the nucleus and cytoplasm. Proc Natl Acad Sci USA 2000;97:12085–12090.
47 Polakis P: The adenomatous polyposis coli (APC) tumor suppressor. Biochim Biophys Acta 1997;1332:F127–F147.
48 Seeling JM, Miller JR, Gil R, Moon RT, White R, Virshup DM: Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science 1999;283:2089–2091.
49 Kawasaki Y, Senda T, Ishidate T, Koyama R, Morishita T, Iwayama Y, Higuchi O, Akiyama T: Asef, a link between the tumor suppressor APC and G-protein signaling. Science 2000;289: 1194–1197.
50 Jimbo T, Kawasaki Y, Koyama R, Sato R, Takada S, Haraguchi K, Akiyama T: Identification of a link between the tumour suppressor APC and the kinesin superfamily. Nat Cell Biol 2002;4:323–327.
51 Kawasaki Y, Sato R, Akiyama T: Mutated APC and Asef are involved in the migration of colorectal tumour cells. Nat Cell Biol 2003;5:211–215.
52 Hughes SA, Carothers AM, Hunt DH, Moran AE, Mueller JD, Bertagnolli MM: Adenomatous polyposis coli truncation alters cytoskeletal structure and microtubule stability in early intestinal tumorigenesis. J Gastrointest Surg 2002;6:868–874; discussion 875.
53 Mahmoud NN, Boolbol SK, Bilinski RT, Martucci C, Chadburn A, Bertagnolli MM: *Apc* gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Res 1997;57:5045–5050.
54 Li Z, Nathke IS: Tumor-associated NH_2-terminal fragments are the most stable part of the adenomatous polyposis coli protein and can be regulated by interactions with COOH-terminal domains. Cancer Res 2005;65:5195–5204.
55 Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P: Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol 1998;8:573–581.
56 Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W: Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 1998;280:596–599.
57 Fagotto F, Jho E, Zeng L, Kurth T, Joos T, Kaufmann C, Costantini F: Domains of axin involved in protein-protein interactions, Wnt pathway inhibition, and intracellular localization. J Cell Biol 1999;145:741–756.
58 Kishida M, Koyama S, Kishida S, Matsubara K, Nakashima S, Higano K, Takada R, Takada S, Kikuchi A: Axin prevents Wnt-3a-induced accumulation of beta-catenin. Oncogene 1999;18: 979–985.

59 Ikeda S, Kishida M, Matsuura Y, Usui H, Kikuchi A: GSK-3beta-dependent phosphorylation of adenomatous polyposis coli gene product can be modulated by beta-catenin and protein phosphatase 2A complexed with Axin. Oncogene 2000;19:537–545.
60 Jiang J, Struhl G: Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature 1998;391:493–496.
61 Marikawa Y, Elinson RP: Beta-TrCP is a negative regulator of Wnt/beta-catenin signaling pathway and dorsal axis formation in *Xenopus* embryos. Mech Dev 1998;77:75–80.
62 Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W: Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 1996;382:638–642.
63 Molenaar M, van de Wetering M, Oosterwegel M, Peterson-Maduro J, Godsave S, Korinek V, Roose J, Destree O, Clevers H: XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. Cell 1996;86:391–399.
64 Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destree O, Clevers H: The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature 1998;395:608–612.
65 Deka J, Kuhlmann J, Muller O: A domain within the tumor suppressor protein APC shows very similar biochemical properties as the microtubule-associated protein tau. Eur J Biochem 1998;253:591–597.
66 Munemitsu S, Souza B, Muller O, Albert I, Rubinfeld B, Polakis P: The *APC* gene product associates with microtubules in vivo and promotes their assembly in vitro. Cancer Res 1994;54: 3676–3681.
67 Smith KJ, Levy DB, Maupin P, Pollard TD, Vogelstein B, Kinzler KW: Wild-type but not mutant APC associates with the microtubule cytoskeleton. Cancer Res 1994;54:3672–3675.
68 Askham JM, Moncur P, Markham AF, Morrison EE: Regulation and function of the interaction between the APC tumour suppressor protein and EB1. Oncogene 2000;19:1950–1958.
69 Dikovskaya D, Newton IP, Nathke IS: The adenomatous polyposis coli protein is required for the formation of robust spindles formed in CSF *Xenopus* extracts. Mol Biol Cell 2004;15: 2978–2991.
70 Matsumine A, Ogai A, Senda T, Okumura N, Satoh K, Baeg GH, Kawahara T, Kobayashi S, Okada M, Toyoshima K, Akiyama T: Binding of APC to the human homolog of the *Drosophila* discs large tumor suppressor protein. Science 1996;272:1020–1023.
71 Henderson BR, Fagotto F: The ins and outs of APC and beta-catenin nuclear transport. EMBO Rep 2002;3:834–839.
72 Deka J, Herter P, Sprenger-Haussels M, Koosch S, Franz D, Muller KM, Kuhnen C, Hoffmann I, Muller O: The APC protein binds to A/T rich DNA sequences. Oncogene 1999;18:5654–5661.
73 Brocardo M, Nathke IS, Henderson BR: Redefining the subcellular location and transport of APC: new insights using a panel of antibodies. EMBO Rep 2005;6:184–190.
74 Nathke IS, Adams CL, Polakis P, Sellin JH, Nelson WJ: The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. J Cell Biol 1996;134:165–179.
75 Rosin-Arbesfeld R, Ihrke G, Bienz M: Actin-dependent membrane association of the APC tumour suppressor in polarized mammalian epithelial cells. EMBO J 2001;20:5929–5939.
76 Mogensen MM, Tucker JB, Mackie JB, Prescott AR, Nathke IS: The adenomatous polyposis coli protein unambiguously localizes to microtubule plus ends and is involved in establishing parallel arrays of microtubule bundles in highly polarized epithelial cells. J Cell Biol 2002;157:1041–1048.
77 Schuyler SC, Pellman D: Microtubule 'plus-end-tracking proteins': the end is just the beginning. Cell 2001;105:421–424.
78 Zumbrunn J, Kinoshita K, Hyman AA, Nathke IS: Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3beta phosphorylation. Curr Biol 2001;11:44–49.
79 Etienne-Manneville S, Hall A: Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature 2003;421:753–756.
80 Barth AI, Siemers KA, Nelson WJ: Dissecting interactions between EB1, microtubules and APC in cortical clusters at the plasma membrane. J Cell Sci 2002;115:1583–1590.
81 Su LK, Burrell M, Hill DE, Gyuris J, Brent R, Wiltshire R, Trent J, Vogelstein B, Kinzler KW: APC binds to the novel protein EB1. Cancer Res 1995;55:2972–2977.

82 Tirnauer JS, Bierer BE: EB1 proteins regulate microtubule dynamics, cell polarity, and chromosome stability. J Cell Biol 2000;149:761–766.
83 Galjart N, Perez F: A plus-end raft to control microtubule dynamics and function. Curr Opin Cell Biol 2003;15:48–53.
84 Berrueta L, Tirnauer JS, Schuyler SC, Pellman D, Bierer BE: The APC-associated protein EB1 associates with components of the dynactin complex and cytoplasmic dynein intermediate chain. Curr Biol 1999;9:425–428.
85 Mimori-Kiyosue Y, Shiina N, Tsukita S: The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules. Curr Biol 2000;10:865–868.
86 Bu W, Su LK: Characterization of functional domains of human EB1 family proteins. J Biol Chem 2003;278:49721–49731.
87 Wen Y, Eng CH, Schmoranzer J, Cabrera-Poch N, Morris EJ, Chen M, Wallar BJ, Alberts AS, Gundersen GG: EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. Nat Cell Biol 2004;6:820–830.
88 Berrueta L, Kraeft SK, Tirnauer JS, Schuyler SC, Chen LB, Hill DE, Pellman D, Bierer BE: The adenomatous polyposis coli-binding protein EB1 is associated with cytoplasmic and spindle microtubules. Proc Natl Acad Sci USA 1998;95:10596–10601.
89 Morrison EE, Wardleworth BN, Askham JM, Markham AF, Meredith DM: EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. Oncogene 1998;17:3471–3477.
90 Honnappa S, John CM, Kostrewa D, Winkler FK, Steinmetz MO: Structural insights into the EB1-APC interaction. EMBO J 2005;24:261–269.
91 Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW: APC mutations occur early during colorectal tumorigenesis. Nature 1992;359:235–237.
92 Su LK, Johnson KA, Smith KJ, Hill DE, Vogelstein B, Kinzler KW: Association between wild type and mutant APC gene products. Cancer Res 1993;53:2728–2731.
93 Jais P, Sabourin JC, Bombled J, Rougier P, Lasser P, Duvillard P, Benard J, Bressac-de Paillerets B: Absence of somatic alterations of the EB1 gene adenomatous polyposis coli-associated protein in human sporadic colorectal cancers. Br J Cancer 1998;78:1356–1360.
94 Smits R, Kielman MF, Breukel C, Zurcher C, Neufeld K, Jagmohan-Changur S, Hofland N, van Dijk J, White R, Edelmann W, Kucherlapati R, Khan PM, Fodde R: Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. Genes Dev 1999;13:1309–1321.
95 Olmeda D, Castel S, Vilaro S, Cano A: Beta-catenin regulation during the cell cycle: implications in G_2/M and apoptosis. Mol Biol Cell 2003;14:2844–2860.
96 Louie RK, Bahmanyar S, Siemers KA, Votin V, Chang P, Stearns T, Nelson WJ, Barth AI: Adenomatous polyposis coli and EB1 localize in close proximity of the mother centriole and EB1 is a functional component of centrosomes. J Cell Sci 2004;117:1117–1128.
97 Tighe A, Johnson VL, Taylor SS: Truncating APC mutations have dominant effects on proliferation, spindle checkpoint control, survival and chromosome stability. J Cell Sci 2004;117:6339–6353.
98 Green RA, Kaplan KB: Chromosome instability in colorectal tumor cells is associated with defects in microtubule plus-end attachments caused by a dominant mutation in APC. J Cell Biol 2003;163:949–961.
99 Taylor SS, Hussein D, Wang Y, Elderkin S, Morrow CJ: Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. J Cell Sci 2001;114:4385–4395.
100 Bloom K: It's a kar9ochore to capture microtubules. Nat Cell Biol 2000;2:E96–E98.
101 Reilein A, Nelson WJ: APC is a component of an organizing template for cortical microtubule networks. Nat Cell Biol 2005;7:463–473.
102 Beach DL, Thibodeaux J, Maddox P, Yeh E, Bloom K: The role of the proteins Kar9 and Myo2 in orienting the mitotic spindle of budding yeast. Curr Biol 2000;10:1497–1506.
103 Liakopoulos D, Kusch J, Grava S, Vogel J, Barral Y: Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment. Cell 2003;112:561–574.
104 Bienz M: The subcellular destinations of APC proteins. Nat Rev Mol Cell Biol 2002;3:328–338.
105 Doxsey S: The centrosome – a tiny organelle with big potential. Nat Genet 1998;20:104–106.

106 Samowitz WS, Powers MD, Spirio LN, Nollet F, van Roy F, Slattery ML: Beta-catenin mutations are more frequent in small colorectal adenomas than in larger adenomas and invasive carcinomas. Cancer Res 1999;59:1442–1444.
107 Mahmoud NN, Bilinski RT, Churchill MR, Edelmann W, Kucherlapati R, Bertagnolli MM: Genotype-phenotype correlation in murine *Apc* mutation: differences in enterocyte migration and response to sulindac. Cancer Res 1999;59:353–359.
108 Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ: Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev 2004;18:1385–1390.
109 Sieber OM, Heinimann K, Gorman P, Lamlum H, Crabtree M, Simpson CA, Davies D, Neale K, Hodgson SV, Roylance RR, Phillips RK, Bodmer WF, Tomlinson IP: Analysis of chromosomal instability in human colorectal adenomas with two mutational hits at APC. Proc Natl Acad Sci USA 2002;99:16910–16915.
110 Tighe A, Johnson VL, Albertella M, Taylor SS: Aneuploid colon cancer cells have a robust spindle checkpoint. EMBO Rep 2001;2:609–614.
111 Dobles M, Liberal V, Scott ML, Benezra R, Sorger PK: Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. Cell 2000;101:635–645.
112 Kalitsis P, Earle E, Fowler KJ, Choo KH: *Bub3* gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. Genes Dev 2000;14:2277–2282.
113 Imai Y, Shiratori Y, Kato N, Inoue T, Omata M: Mutational inactivation of mitotic checkpoint genes, *hsMAD2* and *hBUB1*, is rare in sporadic digestive tract cancers. Jpn J Cancer Res 1999;90: 837–840.
114 Rao CV, Yang YM, Swamy MV, Liu T, Fang Y, Mahmood R, Jhanwar-Uniyal M, Dai W: Colonic tumorigenesis in $BubR1^{+/-}Apc^{Min/+}$ compound mutant mice is linked to premature separation of sister chromatids and enhanced genomic instability. Proc Natl Acad Sci USA 2005;102: 4365–4370.
115 Ee HC, Erler T, Bhathal PS, Young GP, James RJ: Cdx-2 homeodomain protein expression in human and rat colorectal adenoma and carcinoma. Am J Pathol 1995;147:586–592.
116 Mallo GV, Rechreche H, Frigerio JM, Rocha D, Zweibaum A, Lacasa M, Jordan BR, Dusetti NJ, Dagorn JC, Iovanna JL: Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Down-regulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis. Int J Cancer 1997;74:35–44.
117 Aoki K, Tamai Y, Horiike S, Oshima M, Taketo MM: Colonic polyposis caused by mTOR-mediated chromosomal instability in Apc^{+}/Delta716 $Cdx2^{+/-}$ compound mutant mice. Nat Genet 2003;35:323–330.
118 Artandi SE, DePinho RA: A critical role for telomeres in suppressing and facilitating carcinogenesis. Curr Opin Genet Dev 2000;10:39–46.
119 Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 2000;100:57–70.
120 Rudolph KL, Millard M, Bosenberg MW, DePinho RA: Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat Genet 2001;28:155–159.
121 Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC: Telomere reduction in human colorectal carcinoma and with ageing. Nature 1990;346:866–868.
122 Engelhardt M, Drullinsky P, Guillem J, Moore MA: Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer. Clin Cancer Res 1997;3: 1931–1941.
123 Daniels MJ, Wang Y, Lee M, Venkitaraman AR: Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. Science 2004;306:876–879.
124 Cahill DP, Kinzler KW, Vogelstein B, Lengauer C: Genetic instability and darwinian selection in tumours. Trends Cell Biol 1999;9:M57–M60.
125 Carroll PE, Okuda M, Horn HF, Biddinger P, Stambrook PJ, Gleich LL, Li YQ, Tarapore P, Fukasawa K: Centrosome hyperamplification in human cancer: chromosome instability induced by p53 mutation and/or *Mdm2* overexpression. Oncogene 1999;18:1935–1944.
126 Fuchs E, Cleveland DW: A structural scaffolding of intermediate filaments in health and disease. Science 1998;279:514–519.

127 Schliwa M, Euteneuer U, Graf R, Ueda M: Centrosomes, microtubules and cell migration. Biochem Soc Symp 1999;65:223–231.
128 Piel M, Nordberg J, Euteneuer U, Bornens M: Centrosome-dependent exit of cytokinesis in animal cells. Science 2001;291:1550–1553.
129 Griffin CS, Simpson PJ, Wilson CR, Thacker J: Mammalian recombination-repair genes *XRCC2* and *XRCC3* promote correct chromosome segregation. Nat Cell Biol 2000;2:757–761.
130 Shekhar MP, Lyakhovich A, Visscher DW, Heng H, Kondrat N: Rad6 overexpression induces multinucleation, centrosome amplification, abnormal mitosis, aneuploidy, and transformation. Cancer Res 2002;62:2115–2124.
131 Hinchcliffe EH, Sluder G: Centrosome reproduction in *Xenopus* lysates. Methods Cell Biol 2001;67: 269–287.
132 Piel M, Bornens M: Centrosome reproduction in vitro: mammalian centrosomes in *Xenopus* lysates. Methods Cell Biol 2001;67:289–304.
133 Tomonaga T, Matsushita K, Ishibashi M, Nezu M, Shimada H, Ochiai T, Yoda K, Nomura F: Centromere protein H is up-regulated in primary human colorectal cancer and its overexpression induces aneuploidy. Cancer Res 2005;65:4683–4689.
134 Li GQ, Li H, Zhang HF: Mad2 and p53 expression profiles in colorectal cancer and its clinical significance. World J Gastroenterol 2003;9:1972–1975.
135 Michel L, Benezra R, Diaz-Rodriguez E: MAD2 dependent mitotic checkpoint defects in tumorigenesis and tumor cell death: a double edged sword. Cell Cycle 2004;3:990–992.
136 Shiloh Y: ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 2003;3:155–168.
137 Casper AM, Nghiem P, Arlt MF, Glover TW: ATR regulates fragile site stability. Cell 2002;111: 779–789.
138 Smith L, Liu SJ, Goodrich L, Jacobson D, Degnin C, Bentley N, Carr A, Flaggs G, Keegan K, Hoekstra M, Thayer MJ: Duplication of ATR inhibits MyoD, induces aneuploidy and eliminates radiation-induced G_1 arrest. Nat Genet 1998;19:39–46.
139 Kuhn EM: Localization by Q-banding of mitotic chiasmata in cases of Bloom's syndrome. Chromosoma 1976;57:1–11.
140 Goss KH, Risinger MA, Kordich JJ, Sanz MM, Straughen JE, Slovek LE, Capobianco AJ, German J, Boivin GP, Groden J: Enhanced tumor formation in mice heterozygous for *Blm* mutation. Science 2002;297:2051–2053.
141 Moynahan ME, Pierce AJ, Jasin M: BRCA2 is required for homology-directed repair of chromosomal breaks. Mol Cell 2001;7:263–272.
142 Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR, West SC: Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. Mol Cell 2001;7: 273–282.
143 Sanchez C, Perez M, Avila J: GSK3beta-mediated phosphorylation of the microtubule-associated protein 2C (MAP2C) prevents microtubule bundling. Eur J Cell Biol 2000;79:252–260.
144 Goold RG, Owen R, Gordon-Weeks PR: Glycogen synthase kinase 3beta phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. J Cell Sci 1999;112 (Pt 19):3373–3384.
145 Chen Y, Riley DJ, Chen PL, Lee WH: HEC, a novel nuclear protein rich in leucine heptad repeats specifically involved in mitosis. Mol Cell Biol 1997;17:6049–6056.
146 Pei L, Melmed S: Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol Endocrinol 1997;11:433–441.
147 Yu R, Lu W, Chen J, McCabe CJ, Melmed S: Overexpressed pituitary tumor-transforming gene causes aneuploidy in live human cells. Endocrinology 2003;144:4991–4998.
148 Ekholm-Reed S, Spruck CH, Sangfelt O, van Drogen F, Mueller-Holzner E, Widschwendter M, Zetterberg A, Reed SI: Mutation of hCDC4 leads to cell cycle deregulation of cyclin E in cancer. Cancer Res 2004;64:795–800.
149 Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B: Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990;249:912–915.

150 Spruck CH, Won KA, Reed SI: Deregulated cyclin E induces chromosome instability. Nature 1999;401:297–300.
151 O'Hagan RC, Chang S, Maser RS, Mohan R, Artandi SE, Chin L, DePinho RA: Telomere dysfunction provokes regional amplification and deletion in cancer genomes. Cancer Cell 2002;2: 149–155.

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c-Myc, Genomic Instability and Disease

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Abstract

The proto-oncogene c-*myc* has been the subject of intensive research since its discovery. It is already known that this oncogene targets multiple pathways for the initiation and promotion of tumor formation, and that deregulation of this protein is observed in numerous cancers. However, despite the plethora of information gathered, the exact role and mechanism of action of the protein still remains enigmatic. This review focuses on the role of the c-Myc protein in the induction of genomic instability and its link with the development of cancer. We briefly describe c-Myc protein, its binding partners and downstream targets as well as its role in inducing genomic instability and the c-*myc*-related diseases in humans and mice with regard to genomic instability. This review emphasizes the notions that c-Myc is a multifunctional protein which also affects the stability of the whole genome and triggers the initiation of a complex network of genomic instability and therefore acts beyond the characteristics of classical transcription factors that only regulate a limited number of downstream targets. We propose that c-Myc is a structural modifier of the genome that affects the nuclear organization and an important molecule in tumor cell progression through the induction of genomic instability.

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Since c-*myc*'s identification as the normal cellular counterpart of the viral oncogene in avian retroviruses 25 years ago, it has been the subject of intensive research. Numerous studies have established that this oncogene targets multiple cellular pathways for the initiation and promotion of tumor formation, and consequently its deregulation is observed in a wide variety of cancers. Indeed, up to 70% of human cancers show deregulated expression of c-*myc*, placing the c-*myc* gene among the most important human proto-oncogenes. However, despite the vast amount of research on c-*myc*, the exact role and mechanism of action of this oncoprotein still remains enigmatic. c-Myc is a multifunctional protein that can affect the stability of the genome and trigger the initiation of a complex

network of genomic instability. This has been the topic of numerous studies over the last decade and this is still actively pursued.

This review will focus on the role of c-Myc protein in the induction of genomic instability and its link with the development of cancer. These research themes are interconnected and widely studied in many laboratories, therefore this review will be subdivided into three parts: i) Myc, with a brief overview of the gene, the protein and its partners and downstream targets; ii) c-Myc-induced genomic instability; and iii) cancers and other diseases linked to c-*myc*-induced genomic instability in both humans and the mouse.

c-*myc*

The c-*myc* gene is a member of the *myc* gene family that includes three well-characterized members: c-*myc*, N-*myc* and L-*myc*. They share similar genomic organization and the corresponding proteins contain several regions of high sequence homology [1]. There are two additional genes, B-*myc* and s-*myc*, that have been identified only in rodents.

The c-myc Gene

The c-*myc* gene was first isolated as the chicken cellular homologue to the viral oncogene (v-*myc*) of the avian **MY**elo**C**ytomatosis retrovirus [2]. It was subsequently characterized and cloned in different species: human, mouse, rat [3–5], but also in trout, frog, zebrafish, and the sea star [6–9]. A c-*myc* homologue in *Drosophila* was also isolated later [10]. However, no c-*myc* homologue has yet been described from yeast or nematodes. The c-*myc* gene, located on human chromosome 8, on mouse chromosome 15, is composed of three exons: the first is long and non-translated, the other two exons are coding (exons 2 and 3) [11].

The c-Myc Protein

Translation of the major 64 kiloDalton (kDa) polypeptide is initiated at the canonical AUG start codon (exon 2), generating a 439 amino acid protein. A longer (67 kDa) and shorter (45 kDa) polypeptide has also been recognized [12, 13]. The product of the c-*myc* gene is nuclear-located and modified by O-linked glycosylation and phosphorylation, with a half-life of about 20–30 minutes [14]. The c-Myc protein is a transcription factor of the basic-helix-loop-helix-leucine zipper (bHLH-LZ) family [15, 16], whose main function is therefore to regulate transcription of other genes, either inducing or repressing their expression. As a transcription factor, c-Myc is composed of two domains: a DNA-binding domain and a transcription activation domain.

The DNA-binding domain is located in the 90 amino acid carboxy-terminus. This domain is necessary for all c-Myc biological functions [17]. The protein contains two nuclear localization sequences (NLS), and a basic region, implicated in specific DNA sequence recognition and binding at the core DNA consensus site CA(C/T)GTG (the E-box) [1, 16, 18–21]. Immediately downstream of this domain are the contiguous HLH-LZ motifs responsible for specific heterodimer formation between c-Myc and its binding partner, Max [16].

The Transcription Activation Domain (TAD) is also required for all c-Myc biological activities [22]. The TAD contains two Myc boxes, sequences that are highly conserved between the Myc family proteins. Myc box I extends from amino acids 47–62 and Myc box II from amino acids 106–143 [23]. Both c-Myc boxes are essential for the protein's transcriptional activation, repression and also transforming activity [17, 22].

Expression of the c-myc Gene

In general, c-*myc* expression correlates tightly with the proliferation potential of cells [1]. In quiescent cells, c-*myc* expression is virtually undetectable. Upon mitogen or serum stimulation, there is a rapid and transient burst in both c-*myc* mRNA and c-Myc protein expression as cells enter the G_1 phase, followed by a gradual decline to low but detectable steady-state levels in proliferating cells [24]. This induction of c-*myc* transcription occurs in the absence of de novo protein synthesis, indicating that c-myc is an immediate early gene and is directly downstream of mitogenic signaling cascades [25]. Upon growth factor withdrawal or terminal differentiation signals, c-*myc* mRNA and protein decline to nearly undetectable levels in differentiated cells [26].

Regulation of c-Myc

A variety of overlapping control mechanisms cooperate to rapidly and efficiently modulate c-*myc* expression and function in response to internal and external signals. An important regulation of c-Myc protein activity occurs through protein phosphorylation. c-Myc can be phosphorylated at more than a dozen serine and threonine sites, especially within the Myc boxes, by protein kinases like MAPK, Raf/ERK or $p34^{cdk1}$. These phosphorylations can either stabilize the protein or induce its degradation through the ubiquitin/proteasome pathway [27–30]. However, despite its rapid turn-over, metabolically stable and unstable forms of the c-Myc protein coexist in cells, suggesting that the rate of destruction of Myc molecules is linked to their specific functions [31].

The Myc/Max/Mad Network

The identification of Max as binding partner of the Myc family proteins initiated a search for additional bHLH-LZ-based dimerization partners. This led

to the identification of the Mad protein family consisting of Mad1, Mxi1, Mad3 and Mad4. Together these factors define the Myc/Max/Mad network [1, 32]. Other proteins have been added to this network including Mnt, Mga, Mlx, MondoA or WBSCR14 (reviewed in [33]). All of these proteins possess a bHLH-LZ motif, can form dimers and can bind at E-box DNA sequences suggesting that they function as transcriptional regulators [32, 34–36]. Only c-Myc proteins, MondoA and WBSCR14 carry a TAD and can therefore be implicated in target gene transactivation. In contrast to Myc, Mad proteins and Mnt possess an mSin3 interaction domain which mediates transcriptional repression [37–39]. A gene repression activity has also been attributed to c-Myc itself [32, 40, 41]. One mechanism of c-Myc-dependent repressor function is through blocking of the positive acting initiator (INR) binding factor Miz-1 [42–44].

Proteins from the Myc and Mad families are rapidly synthesized in response to mitogenic or differentiation signals, whereas Max and other co-activators or co-repressors are stable and constitutively expressed. This suggests that the Myc/Max/Mad network of proteins triggers the opposite transcriptional control of a common set of target genes through the relative abundance of the Max-associated transcription factors (for review, see [32]).

c-Myc-Mediated Gene Expression Regulation: Chromatin Remodeling

Gene expression regulation by the Myc and Max proteins occurs through two types of chromatin remodeling mechanisms: i) histone acetylation, controlled by histone acetyl-transferases (HATs) and histone deacetylases (HDACs) [38, 45–47] and ii) ATP-dependent remodeling of nucleosomes, catalyzed by large protein assemblages such as the SWI/SNF complex [45, 48]. These complexes are recruited to specific promoters via their association with DNA-bound activators (i.e. Myc proteins) or repressors (i.e. Mad proteins). c-Myc associates with both HAT and SWI/SNF-type complexes through the TRRAP [49] and SNF5 [50] proteins, respectively. It was therefore shown that upon binding to DNA in vivo, c-Myc rapidly induced histone acetylation near E-boxes of various target genes [51]. In addition, it is known that c-Myc is recruited to the core promoters through other protein-protein interactions, like with TFII-I, YY-1, Sp-1, Smad-2 and -3, NF-Y or Miz-1 [44]. On the other hand, the repressive action of the Mad/Mxi proteins via HDACs is well described [1, 32, 37, 38]. Thus the Myc/Max/Mad complexes function as an acetylase/deacetylase switch to transcriptionally regulate a wide range of RNA polymerase II-transcribed genes (for review, see [41]). In addition, it was shown that c-Myc interacts with the RNA polymerase III apparatus to enhance transcription of transfer RNAs, 5S ribosomal RNA and a subset of other small RNA genes, through its association with the TFIIIB complex [52]. More recently, three studies showed that c-Myc also enhances directly ribosomal RNA synthesis by RNA polymerase I in addition to controlling

RNA polymerase II- and III-regulated gene transcription, suggesting that Myc promotes the generation of crucial components of a functional ribosome [53–56], and plays a role also in the regulation of translation [57].

Target Genes

A wide variety of techniques has been employed in the hunt for c-Myc targets, ranging from differential expression assays or promoter analysis to the modern methods of microarray profiling, serial analysis of gene expression or chromatin immunoprecipitation [35, 51, 58–70]. A high number of c-Myc target genes has now been identified. However, the different groups of investigators have used various approaches to identify c-Myc targets. These include the induction of c-Myc targets using a c-Myc-estrogen receptor hybrid protein or other fusion proteins, comparisons of c-Myc knockout cells with c-Myc wild-type cells, or various strategies for supplying c-Myc encoded in vectors or interfere with c-Myc functions [15, 71, 72]. Although there is some overlap among the c-Myc-regulated genes identified, considerable differences also occur. Nevertheless, large scale analysis of targets at the genomic level revealed that the extent of transcriptional changes that can be triggered by Myc is remarkable and involves thousands of genes. The heterodimer c-Myc/Max was estimated to occupy more than 15% of the gene promoters tested in Burkitt's lymphoma cells [58]. Altogether, the consensus seems to indicate that c-Myc-activated functions are indicative of a physiological state geared toward the rapid utilization of carbon sources, the biosynthesis of precursors for macromolecular synthesis and the accumulation of cellular mass. In contrast, the majority of Myc-repressed genes are involved in the interactions and communications of cells with their external environment and several are known to possess antiproliferative or antimetastatic properties [62]. Hence, the genes regulated by c-Myc are believed to elicit the variety of biological and physiological effects of the protein, including cell division, cell growth and proliferation, metabolism, apoptosis, angiogenesis and adhesion, immortalization and stem/progenitor cell differentiation (for review, see [32, 73–75]).

Genomic Instability

Recently, a dozen microarray-based screens have added over 600 genes to the list of potential c-Myc targets, without regard to whether these are direct or indirect targets. However, other studies have surprisingly shown that c-Myc only alters the transcription level of a minority of the genes that it binds to. This led to the hypothesis that c-Myc could function as a widespread regulator of transcription in contrast to the classical transcription factor that regulates a

limited number of genes downstream of its binding site [76]. This suggests a more global function for c-Myc at the global DNA level, such as regulation of genomic structural integrity or repair or replication of DNA.

In fact, we and others have already described non-transcriptional roles for c-Myc. We and others have previously shown a role for c-Myc in the DNA replication process and more importantly that deregulated c-Myc expression generates genomic instability. This is manifested by gene amplification (both intra- and extrachromosomally), gene rearrangements, karyotypic instability, and overall disruption of the three-dimensional structure of the nucleus [77, 78].

What is Genomic Instability?

The term genomic instability refers to genetic changes that affect the normal organization and function of genes and chromosomes. These alterations can be structural or numerical. Structural genomic instability encompasses point mutations, deletions, microsatellite instability, duplications, amplifications, translocations and inversions. Numerical genomic instability on the other hand, also called aneuploidy, corresponds to aberrations in the number of chromosomes in each cell, and extends from nullisomy to polysomy. The simultaneous combination of both structural and numerical genomic instability within a cell is called karyotypic instability or chromosomal instability.

c-Myc induces a complex network of genomic instability that can include several components: locus-specific genomic instability, karyotypic or chromosomal instability, long-range illegitimate recombination, point mutations and DNA breakage (for review, see [77]).

Replication

A role for c-Myc in DNA replication was initially suggested for the replication of the Simian Virus 40. It was shown that overexpression of the c-*myc* gene product allows the replication of SV40 in human lymphoma cells and that c-Myc protein might be associated with the DNA-protein replication complex [79, 80]. Other studies showed that c-Myc was able to promote endoreduplication in the presence of a mitotic block [81]. Upregulation of c-*myc* was also shown to cause endoreduplication in keratinocytes prior to their terminal differentiation [82] and we have shown that c-Myc initiates illegitimate replication of the ribonucleotide reductase R2 gene [83]. This ability of c-Myc to induce illegitimate rounds of replication is the basis of one of the mechanisms through which c-Myc induces gene amplification.

Locus-Specific Genomic Instability

c-Myc induces the instability of some genes, but not others, leading to the generation of 'locus-specific' genomic instability. The genes specifically targeted

by this c-Myc-induced genomic instability do not correspond perfectly to c-Myc transcriptional targets, and discrepancies between 'instability targets' and 'transcription targets' exist. This type of locus-specific instability was first described for the dihydrofolate reductase (*DHFR*) gene that was amplified as a result of c-*myc* inducible overexpression [84, 85]. At the same time, genes such as cyclin C, ribonucleotide reductase R1, syndecan-1, and glyceraldehyde dehydrogenase (*GAPDH*) were not affected in their genomic stability. *DHFR* was amplified within 72 hours of c-Myc deregulation in a variety of cell lines of mouse, hamster and human origin, and in several tissue types. The amplification of *DHFR* was reversible when c-Myc was induced transiently [85].

In vivo studies subsequently showed that *DHFR* was amplified in primary mouse plasmacytomas, Myc-dependent B lymphocytic tumors that develop in susceptible mice, such as BALB/c (for review, see [86]). Plasmacytoma induction studies showed that *DHFR* was amplified within the first week of c-*myc* deregulation [87]. The amplification of the *DHFR* gene occurred intra- and extrachromosomally in mouse and human cells, however only intrachromosomally in the hamster cell line CHO-9 [88]. Subsequently, additional genes were shown to lose stability when c-*myc* expression was deregulated. Among them were: ribonucleotide reductase R2 [89], cyclin D2 [90], *ODC* and *CAD* [91]. In addition, cyclin B1 was shown to be co-amplified with *DHFR*, probably due to the close proximity of the two genes [92]. Altogether, the number of genes known to be destabilized by c-Myc is so far small, but it will undoubtedly increase with the use of high-throughput analysis methods. However, all of these genes are amplified both intrachromosomally and extrachromosomally in the presence of inducible or constitutive c-*myc* expression. Nevertheless, the resulting increase in gene dosage remains low. Usually, c-Myc deregulation leads to the formation of three to four additional copies of the target genes.

Extrachromosomal Elements

Using inducible vectors, we have shown that c-Myc induces the formation of extrachromosomal elements (EEs) in vitro [89, 90]. EEs refer to all extrachromosomal DNA molecules found in the cell, irrespective of their transcriptional and/or replicational potential. The size of such EEs varies from 0.01 to 2 μm. Small EEs contain only repetitive sequence motifs (as found in normal cells) whereas the large EEs including episomes and 'double minute chromosomes' contain genes and are found in tumors. EEs have been found in all organisms analyzed so far (frog, fly, rodent, human). Their role, if any, and mechanism of formation are still essentially unknown (for review, see [93]). EEs have been correlated with a malignant phenotype by studies in cell lines [94–97] and tumor cells often harbor EEs [98]. Some of the genes found on EEs

include oncogenes, such as c-*myc* itself or drug resistance genes (for review, see [77, 99]).

The c-Myc-dependent induction of extra-chromosomally amplified genes is a novel pathway of c-Myc-dependent genomic instability. The genes found on these EEs carry histone proteins, are able to replicate, and some of them are transcriptionally active [85, 100, 101]. Also, we showed that c-Myc-induced EEs are generated from all chromosomes, and from different loci on each chromosome (unpublished data). Thus, these EEs behave as functional genetic units that may play a role in cellular transformation. This was demonstrated by our finding that EEs carrying both c-*myc* and Immunoglobulin (*Ig*) H genes were found in a chromosomal translocation-negative plasmacytoma [101]. Since there were no other possible genetic mechanisms that could be responsible for c-*myc* activation in these cells, therefore these c-*myc*/*IgH*-carrying EEs appear to be responsible for c-*myc* deregulation and the subsequent neoplastic transformation of this tumor. Another example of extrachromosomal deregulation of an oncogene was recently shown for c-*abl* in some cases of T-cell acute lymphoblastic leukemia [102]. The generation of these EEs is thought to occur via illegitimate replication, DNA breakage and/or DNA recombination.

Karyotypic Instability

c-*myc* deregulation also induces karyotypic instability [85, 103]. This form of genomic instability was found in cell lines after prolonged c-*myc* deregulation [104]. In addition, when injected into nude mice, these cells, constitutively expressing c-*myc*, developed karyotypic instability [103]. The types of instability seen in vivo were identical to those seen in vitro and include aneuploidy, telomere-centromere fusions, chromosome breakage, formation of unstable ring chromosomes and generation of EEs.

Recombination

c-*myc* deregulation induces recombination events that involve many chromosomes [105]. Such illegitimate recombinations include translocations, deletions and inversions. DNA sequencing and spectral karyotyping have been used to show that a wide variety of chromosomal regions are affected with different break points involved. Further, recent studies have tested whether c-*myc* deregulation also increases the rate of point mutations or large-scale rearrangements [105–107]. However, the results were not concordant between the different studies, probably due to the use of different cell systems.

DNA Breakage

c-Myc has been recently linked directly to the initiation of genomic instability. Indeed, it was shown that brief c-Myc activation could induce DNA damage

prior to S phase in normal human fibroblasts. Damage correlated with the generation of reactive oxygen species without induction of apoptosis [108]. It is known that such DNA breaks are sufficient to induce gene amplifications, deletions and rearrangements [109], leading to loss of heterozygosity [110] and translocations [111]. Furthermore, it was proposed that c-Myc triggers this genomic instability through the generation of DNA breakage while bypassing tumor suppressor *p53* function [85, 104, 108]. Thus c-Myc activation can induce DNA damage and override damage controls, thereby accelerating tumor progression via genomic instability.

3D-Organization of the Nucleus

Recently, we showed that c-Myc deregulation affects the three-dimensional global organization of the mammalian interphase nucleus. This leads to a remodeling of nuclear organization of telomeres and chromosomes, and favors the topological conditions that can initiate genomic instability. Indeed, we described a novel pathway for c-Myc-dependent genomic instability showing that deregulation of c-Myc initiates genomic instability through Bridge-Breakage-Fusion cycles of chromosomes, that was preceded by telomere aggregation and/or fusion in the interphase nucleus [78]. These early events preceding chromosomal rearrangements could be used for the early detection of pre-malignant cells.

Thus, c-Myc can contribute to cancer, during its initiation and progression, through the induction of genomic instability in critical genes and through genome-wide chromosomal rearrangements, acting as a structural modifier of the genome and as a promoter of neoplastic transformation.

Cancer and Other Disease Induction

Early in vitro studies showed that overexpression of the normal c-*myc* gene can induce cellular transformation, either alone (in immortalized rat fibroblasts) [72] or in combination with other cooperating oncogenes (in primary mouse or rat fibroblasts) [112, 113]. It is now known however that c-Myc overexpression in primary mouse embryo fibroblasts tends to result in cell cycle arrest or apoptosis and only cells that survive this crisis become immortalized by c-Myc [114]. The activation of the gene encoding the telomerase subunit (TERT) by c-Myc suggests a possible mechanism for c-Myc-mediated immortalization. However, c-Myc and Ras are not sufficient to transform human primary cells [115]. Human primary cells require ectopic expression of hTERT for neoplastic transformation by activated *ras* and SV40T antigen genes. The difference in the susceptibility of rodent vs. human cells to c-Myc and Ras transformation may reflect the propensity for rodent cells to maintain

telomerase activity and have much longer telomeres. The same synergetic action was recently described for mammary tumorigenesis models, in which c-*myc* can cooperate with *ras* [116] or *met* [117] to transform primary mouse mammary epithelial cells. On the other hand, the expression of the c-*myc* oncogene was sufficient to immortalize primary human prostate epithelial cells [118].

Cancer is a genetic disease, and tumorigenesis is well described as the consequence of alterations in three types of genes: oncogenes, tumor-suppressor genes and stability genes. Researchers have been speculating that genomic instability might be involved in cancer development for many decades [119]. However, the question whether genomic instability is a cause or a consequence of the transformation process is still under intense debate [120]. Genetic instability clearly contributes to cancer, but chromosomal instability is more commonly found in cancers at the whole chromosomal level [121, 122]. Aneuploidy is consistently observed in virtually all cancers [123]. However, while breast, bladder, kidney and ovarian cancers exhibit substantial aneuploidy (68–90%), prostate and colorectal cancers only show 15–22%, suggesting the involvement of different pathways and mechanisms (for review, see [121, 123]). Globally, numerical chromosomal changes (i.e. karyotypic, chromosomal instability, aneuploidy) occur in the vast majority of solid tumors and result in changes in gene dosage (for review, see [124, 125]). The link between genomic instability and neoplasia is also well established in the lymphoid lineage (reviewed in [126]).

On the other hand, c-*myc* is a well known protooncogene and the constitutive expression of its mRNA and protein was recognized as a transformation-initiating event in B-cell lymphomas of humans (Burkitt's lymphoma) [127], rats (immunocytomas in Louvain-strain rats) [128], and mice (oil-induced BALB/c plasmacytomas) [129]. In these tumors, chromosomal translocations juxtaposed the c-*myc* gene to one of the *Ig* heavy or light chain loci. In these cases, the strong enhancers on the *Ig*-encoding genes were responsible for a constitutive activation of c-*myc* transcription [130]. Extrachromosomal translocation of c-*myc* and an *Ig* gene was also described in a murine plasmacytoma [101].

Mouse Models

The development of methods to generate transgenic mouse models that exhibit overexpression of oncogenes has permitted the examination of their function in vivo. The classic experiments of Adams et al. showed that transgenic mice expressing a c-*myc* gene under the control of *Ig* μ or κ enhancer elements develop monoclonal or oligoclonal early pre-B cell lymphoid malignancies [131]. The Eμ-*myc* transgenic mouse model was then developed which mimics the translocated c-*myc* gene found in lymphoid tumors [132]. A marked synergy

between c-*myc* and *bcl*-2 was described in a doubly transgenic mouse model, where hyperproliferation of pre-B and B-cells and tumor development occur much faster than in Eμ-*myc* alone [133]. In the same manner, a synergy was described between c-*myc* and Bcl-X_L to cause plasma cell neoplasms in mice [134]. More recently, the development of mouse models that exhibit conditional activation of oncogenes allowed one to assess the consequences of oncogene inactivation for the maintenance of an established neoplastic phenotype. It was shown that the c-Myc tumorigenesis was dependent on the continuous presence of c-Myc, since c-Myc-initiated tumorigenesis was reversible as long as no additional genetic changes occurred [103, 135–140]. This was described for fibroblasts, osteocytes, lymphocytes and hematopoietic lineages. However, contrasting results have been described in other cellular contexts: pancreatic islets, skin epidermis, keratinocytes and mammary epithelium [141–145]. Other approaches mimicking the translocated c-*myc* allele in the *Ig* locus have been used, for instance gene targeting or knock-in, and have shown the development of B-cell, plasma-cell and Burkitt-like lymphomas in mice [146, 147].

In our laboratory, we have used the interleukine 3-dependant murine pro-B Ba/F3 cell model expressing c-Myc proteins to assess the link between genomic instability and tumorigenesis induced by c-Myc. We showed that overexpression of c-Myc induced structural and numerical genomic instability in this model, as well as in vivo tumorigenesis in SCID mice. A c-Myc box II-deleted mutant protein was also a potent inducer of genomic instability but did not trigger tumorigenesis, showing that the genomic instability phenotype induced by the deleted c-Myc protein can be genetically uncoupled from its tumorigenic potential [148, 149]. These results suggest also that the high mutation rate in c-Myc proteins in Burkitt's lymphomas (more than 60% of mutated proteins) might be the result of secondary events, i.e. somatic hypermutation, that occurred once the oncogenic lesions were already initiated by a wild-type c-Myc protein.

Human Disease

c-*myc* overexpression is associated with neoplasms of different human tissues: breast and cervical carcinoma, neuroblastoma, malignant melanoma, prostate and gastrointestinal cancers, osteogenic sarcoma and lymphoid cancers (for review, see [150]). In lymphoid tumors, c-*myc* is mainly deregulated through chromosomal translocations: the c-*myc* gene is translocated to one of the *Ig* loci in virtually all Burkitt's lymphomas. But c-*myc* gene amplification is also a mechanism of the gene deregulation, and this is the case in a portion of human breast, lung, ovarian and prostate carcinomas, as well as neuroblastoma. For example, it has been described that overreplication and bridge-breakage-fusion-type mechanisms are involved in the genomic instability associated with

human papillomavirus-linked cervical cancers, leading to the amplification of c-*myc* [151]. The same mechanisms of chromosomal instability and gene-amplification have been described in osteosarcomas, in relation with c-*myc* amplification [152, 153]. On the other hand, amplification of the c-*myc*-related gene N-*myc* is a well described characteristic of neuroblastomas and remains the most widely accepted predictive parameter of long-term disease-free survival [150].

It was proposed recently that another potential contribution of c-Myc to the genesis of lymphoma occurs through its ability to serve as a surrogate for cytokines, suggesting that c-Myc plays a critical role in the response of B cells to antigen [154].

Deregulated c-*myc* expression is often associated with aggressive, poorly differentiated tumors. However, given that most human tumors are quite advanced at the time of discovery, often possessing many genetic alterations, it is difficult to ascertain at which stage of tumor progression oncogenic c-*myc* activation occurred.

c-Myc has been linked to other human diseases than cancer such as diabetes. Indeed, c-Myc has been implicated in the loss and dysfunction of insulin-producing β-cells. It appears as a powerful trigger for β-cell apoptosis as well as loss of differentiation in rodent islets in vivo [155, 156]. This suggests that c-Myc might be a key contributor to the development of this disease, not only through its role in deregulation of cell proliferation, but also by virtue of its opposing role in stimulating apoptosis.

Perspective/Conclusion

c-Myc is overexpressed in many human tumor types and has been shown to regulate cell functions that are required for tumorigenesis. However, the target gene(s), if any, that could recapitulate c-Myc's functions, especially in transformation and tumorigenesis, are still missing. It was recently proposed that two c-Myc targets, MT-MC1 (a nuclear protein with homology to certain DNA helicases) and HMG-I (a chromatin modifying nuclear protein of the high-mobility group) are able to recapitulate multiple c-Myc phenotypes, although not all. This suggested that despite the myriad of targets under the control of c-Myc, only a small number are critical for achieving its most important properties with respect to disease [157]. However, c-Myc is a versatile protein, and none of its known targets is able to completely substitute for all of its functions. On the other hand, recent studies have shown that c-Myc binds to approximately 15% of all genes, without clear correlation with the expression of bound genes. This leads to the consideration that c-Myc is a widespread regulator of transcription

and this could be due to c-Myc having a more general action at the genome level. The overall promotion of genomic instability by c-Myc suggests that it may have a central role in destabilizing the genome, and does not act only as a classical transcription factor that regulates a limited number of downstream targets. Consistent with this proposal we recently described a novel pathway of c-Myc-dependent genomic instability in which c-Myc caused a remodeling of the telomere and chromosome organization which precedes the onset of genomic instability and subsequently leads to chromosomal rearrangements [78].

Therefore, we propose that c-Myc is a structural modifier of the genome that could affect the nuclear organization and is an important progressor molecule in neoplastic transformation through the induction of genomic instability.

Investigations of the role of c-Myc in disease development will likely continue to be an important area of scientific research. However, the challenge is now to apply the new techniques of large-scale analysis at the genomic level, in combination with the new imaging technologies, especially the three-dimensional approaches, to further our understanding of c-Myc functions in cancer, as well as in normal cellular processes. It should be expected that improved understanding of the many roles this gene plays in cancer will lead to novel and highly specific therapeutic approaches for the treatment of many cancer types.

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References

1 Henriksson M, Luscher B: Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv Cancer Res 1996;68:109–182.
2 Vennstrom B, Sheiness D, Zabielski J, Bishop JM: Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J Virol 1982;42:773–779.
3 Dalla-Favera R, Gelmann EP, Martinotti S, Franchini G, Papas TS, Gallo RC, Wong-Staal F: Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc Natl Acad Sci USA 1982;79:6497–6501.
4 Stanton LW, Fahrlander PD, Tesser PM, Marcu KB: Nucleotide sequence comparison of normal and translocated murine c-myc genes. Nature 1984;310:423–425.
5 Hayashi K, Makino R, Kawamura H, Arisawa A, Yoneda K: Characterization of rat c-myc and adjacent regions. Nucleic Acids Res 1987;15:6419–6436.
6 King MW, Roberts JM, Eisenman RN: Expression of the c-myc proto-oncogene during development of *Xenopus laevis*. Mol Cell Biol 1986;6:4499–4508.

7 Van Beneden RJ, Watson DK, Chen TT, Lautenberger JA, Papas TS: Cellular myc (c-*myc*) in fish (rainbow trout): its relationship to other vertebrate *myc* genes and to the transforming genes of the MC29 family of viruses. Proc Natl Acad Sci USA 1986;83:3698–3702.
8 Walker CW, Boom JD, Marsh AG: First non-vertebrate member of the myc gene family is seasonally expressed in an invertebrate testis. Oncogene 1992;7:2007–2012.
9 Schreiber-Agus N, Torres R, Horner J, Lau A, Jamrich M, DePinho RA: Comparative analysis of the expression and oncogenic activities of *Xenopus* c-, N-, and L-*myc* homologs. Mol Cell Biol 1993;13:2456–2468.
10 Gallant P, Shiio Y, Cheng PF, Parkhurst SM, Eisenman RN: Myc and Max homologs in *Drosophila*. Science 1996;274:1523–1527.
11 Battey J, Moulding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G, Leder P: The human c-*myc* oncogene: structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell 1983;34:779–787.
12 Hann SR, King MW, Bentley DL, Anderson CW, Eisenman RN: A non-AUG translational initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. Cell 1988;52:185–195.
13 Spotts GD, Patel SV, Xiao Q, Hann SR: Identification of downstream-initiated c-Myc proteins which are dominant-negative inhibitors of transactivation by full-length c-Myc proteins. Mol Cell Biol 1997;17:1459–1468.
14 Luscher B, Eisenman RN: New light on Myc and Myb. Part I. Myc Genes Dev 1990;4:2025–2035.
15 Mateyak MK, Obaya AJ, Adachi S, Sedivy JM: Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ 1997;8:1039–1048.
16 Blackwood EM, Eisenman RN: Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 1991;251:1211–1217.
17 Stone J, de Lange T, Ramsay G, Jakobovits E, Bishop JM, Varmus H, Lee W: Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol Cell Biol 1987;7:1697–1709.
18 Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H: Sequence-specific DNA binding by the c-Myc protein. Science 1990;250:1149–1151.
19 Amati B, Dalton S, Brooks MW, Littlewood TD, Evan GI, Land H: Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature 1992;359:423–426.
20 Blackwell TK, Huang J, Ma A, Kretzner L, Alt FW, Eisenman RN, Weintraub H: Binding of myc proteins to canonical and noncanonical DNA sequences. Mol Cell Biol 1993;13:5216–5224.
21 Solomon DL, Amati B, Land H: Distinct DNA binding preferences for the c-Myc/Max and Max/Max dimers. Nucleic Acids Res 1993;21:5372–5376.
22 Kato GJ, Barrett J, Villa-Garcia M, Dang CV: An amino-terminal c-Myc domain required for neoplastic transformation activates transcription. Mol Cell Biol 1990;10:5914–5920.
23 Ingvarsson S: The myc gene family proteins and their role in transformation and differentiation. Semin Cancer Biol 1990;1:359–369.
24 Kelly K, Cochran BH, Stiles CD, Leder P: Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell 1983;35:603–610.
25 Hann SR, Thompson CB, Eisenman RN: c-myc oncogene protein synthesis is independent of the cell cycle in human and avian cells. Nature 1985;314:366–369.
26 Lemaitre JM, Buckle RS, Mechali M: c-Myc in the control of cell proliferation and embryonic development. Adv Cancer Res 1996;70:95–144.
27 Seth A, Alvarez E, Gupta S, Davis RJ: A phosphorylation site located in the NH_2-terminal domain of c-Myc increases transactivation of gene expression. J Biol Chem 1991;266:23521–23524.
28 Henriksson M, Bakardjiev A, Klein G, Luscher B: Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential. Oncogene 1993;8:3199–3209.
29 Pulverer BJ, Fisher C, Vousden K, Littlewood T, Evan G, Woodgett JR: Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. Oncogene 1994;9:59–70.
30 Sears RC, Nevins JR: Signaling networks that link cell proliferation and cell fate. J Biol Chem 2002;277:11617–11620.
31 Tworkowski KA, Salghetti SE, Tansey WP: Stable and unstable pools of Myc protein exist in human cells. Oncogene 2002;21:8515–8520.

32 Grandori C, Cowley SM, James LP, Eisenman RN: The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 2000;16:653–699.
33 Luscher B: Function and regulation of the transcription factors of the Myc/Max/Mad network. Gene 2001;277:1–14.
34 Dang CV, Resar LM, Emison E, Kim S, Li Q, Prescott JE, Wonsey D, Zeller K: Function of the c-Myc oncogenic transcription factor. Exp Cell Res 1999;253:63–77.
35 Dang CV: c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 1999;19:1–11.
36 Grinberg AV, Hu CD, Kerppola TK: Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells. Mol Cell Biol 2004;24:4294–4308.
37 Schreiber-Agus N, DePinho RA: Repression by the Mad(Mxi1)-Sin3 complex. Bioessays 1998;20:808–818.
38 Knoepfler PS, Eisenman RN: Sin meets NuRD and other tails of repression. Cell 1999;99:447–450.
39 Kiermaier A, Eilers M: Transcriptional control: calling in histone deacetylase. Curr Biol 1997;7: R505–R507.
40 Claassen GF, Hann SR: Myc-mediated transformation: the repression connection. Oncogene 1999;18:2925–2933.
41 Amati B, Frank SR, Donjerkovic D, Taubert S: Function of the c-Myc oncoprotein in chromatin remodeling and transcription. Biochim Biophys Acta 2001;1471:M135–M145.
42 Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massague J: TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. Nat Cell Biol 2001;3:400–408.
43 Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Moroy T, Bartek J, Massague J, Hanel F, Eilers M: Repression of p15INK4b expression by Myc through association with Miz-1. Nat Cell Biol 2001;3:392–399.
44 Wanzel M, Herold S, Eilers M: Transcriptional repression by Myc. Trends Cell Biol 2003;13: 146–150.
45 Kingston RE, Narlikar GJ: ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev 1999;13:2339–2352.
46 Sterner DE, Berger SL: Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 2000;64:435–459.
47 Brown CE, Lechner T, Howe L, Workman JL: The many HATs of transcription coactivators. Trends Biochem Sci 2000;25:15–19.
48 Vignali M, Hassan AH, Neely KE, Workman JL: ATP-dependent chromatin-remodeling complexes. Mol Cell Biol 2000;20:1899–1910.
49 McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD: The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 1998;94:363–374.
50 Cheng SW, Davies KP, Yung E, Beltran RJ, Yu J, Kalpana GV: c-MYC interacts with INI1/hSNF5 and requires the SWI/SNF complex for transactivation function. Nat Genet 1999;22:102–105.
51 Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, Greene J, Cocito A, Amati B: Genomic targets of the human c-Myc protein. Genes Dev 2003;17:1115–1129.
52 Gomez-Roman N, Grandori C, Eisenman RN, White RJ: Direct activation of RNA polymerase III transcription by c-Myc. Nature 2003;421:290–294.
53 Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, White RJ: c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol 2005;7:311–318.
54 Grewal SS, Li L, Orian A, Eisenman RN, Edgar BA: Myc-dependent regulation of ribosomal RNA synthesis during *Drosophila* development. Nat Cell Biol 2005;7:295–302.
55 Arabi A, Wu S, Ridderstrale K, Bierhoff H, Shiue C, Fatyol K, Fahlen S, Hydbring P, Soderberg O, Grummt I, Larsson LG, Wright AP: c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nat Cell Biol 2005;7:303–310.
56 Oskarsson T, Trumpp A: The Myc trilogy: lord of RNA polymerases. Nat Cell Biol 2005;7:215–217.
57 Schmidt EV: The role of c-myc in regulation of translation initiation. Oncogene 2004;23: 3217–3221.
58 Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, Ren B: A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc Natl Acad Sci USA 2003;100:8164–8169.

59 Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, Golub TR: Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. Proc Natl Acad Sci USA 2000;97:3260–3265.
60 Menssen A, Hermeking H: Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. Proc Natl Acad Sci USA 2002;99:6274–6279.
61 Watson JD, Oster SK, Shago M, Khosravi F, Penn LZ: Identifying genes regulated in a Myc-dependent manner. J Biol Chem 2002;277:36921–36930.
62 O'Connell BC, Cheung AF, Simkevich CP, Tam W, Ren X, Mateyak MK, Sedivy JM: A large scale genetic analysis of c-Myc-regulated gene expression patterns. J Biol Chem 2003;278: 12563–12573.
63 Haggerty TJ, Zeller KI, Osthus RC, Wonsey DR, Dang CV: A strategy for identifying transcription factor binding sites reveals two classes of genomic c-Myc target sites. Proc Natl Acad Sci USA 2003;100:5313–5318.
64 Orian A, van Steensel B, Delrow J, Bussemaker HJ, Li L, Sawado T, Williams E, Loo LW, Cowley SM, Yost C, Pierce S, Edgar BA, Parkhurst SM, Eisenman RN: Genomic binding by the *Drosophila* Myc, Max, Mad/Mnt transcription factor network. Genes Dev 2003;17:1101–1114.
65 Mao DY, Watson JD, Yan PS, Barsyte-Lovejoy D, Khosravi F, Wong WW, Farnham PJ, Huang TH, Penn LZ: Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. Curr Biol 2003;13:882–886.
66 Schlosser I, Holzel M, Murnseer M, Burtscher H, Weidle UH, Eick D: A role for c-Myc in the regulation of ribosomal RNA processing. Nucleic Acids Res 2003;31:6148–6156.
67 Marinkovic D, Marinkovic T, Kokai E, Barth T, Moller P, Wirth T: Identification of novel Myc target genes with a potential role in lymphomagenesis. Nucleic Acids Res 2004;32:5368–5378.
68 Thomas-Tikhonenko A, Viard-Leveugle I, Dews M, Wehrli P, Sevignani C, Yu D, Ricci S, el-Deiry W, Aronow B, Kaya G, Saurat JH, French LE: Myc-transformed epithelial cells down-regulate clusterin, which inhibits their growth in vitro and carcinogenesis in vivo. Cancer Res 2004;64: 3126–3136.
69 Tsuneoka M, Teye K, Arima N, Soejima M, Otera H, Ohashi K, Koga Y, Fujita H, Shirouzu K, Kimura H, Koda Y: A novel MYC-target gene, mimitin, that is involved in cell proliferation of esophageal squamous cell carcinoma. J Biol Chem 2005;280:19977–19985.
70 Schlosser I, Holzel M, Hoffmann R, Burtscher H, Kohlhuber F, Schuhmacher M, Chapman R, Weidle UH, Eick D: Dissection of transcriptional programmes in response to serum and c-Myc in a human B-cell line. Oncogene 2005;24:520–524.
71 Hermeking H, Rago C, Schuhmacher M, Li Q, Barrett JF, Obaya AJ, O'Connell BC, Mateyak MK, Tam W, Kohlhuber F, Dang CV, Sedivy JM, Eick D, Vogelstein B, Kinzler KW: Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci USA 2000;97:2229–2234.
72 Eilers M, Picard D, Yamamoto KR, Bishop JM: Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature 1989;340:66–68.
73 Levens DL: Reconstructing MYC. Genes Dev 2003;17:1071–1077.
74 Wilson A, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM, Pasche AC, Knabenhans C, Macdonald HR, Trumpp A: c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev 2004;18:2747–2763.
75 Pelengaris S, Khan M: The many faces of c-MYC. Arch Biochem Biophys 2003;416:129–136.
76 Patel JH, Loboda AP, Showe MK, Showe LC, McMahon SB: Analysis of genomic targets reveals complex functions of MYC. Nat Rev Cancer 2004;4:562–568.
77 Mai S, Mushinski JF: c-Myc-induced genomic instability. J Environ Pathol Toxicol Oncol 2003;22:179–199.
78 Louis SF, Vermolen BJ, Garini Y, Young IT, Guffei A, Lichtensztejn Z, Kuttler F, Chuang TC, Moshir S, Mougey V, Chuang AY, Kerr PD, Fest T, Boukamp P, Mai S: c-Myc induces chromosomal rearrangements through telomere and chromosome remodeling in the interphase nucleus. Proc Natl Acad Sci USA 2005;102:9613–9618.
79 Classon M, Henriksson M, Sumegi J, Klein G, Hammarskjold ML: Elevated c-myc expression facilitates the replication of SV40 DNA in human lymphoma cells. Nature 1987;330:272–274.
80 Classon M, Wennborg A, Henriksson M, Klein G: Analysis of c-Myc domains involved in stimulating SV40 replication. Gene 1993;133:153–161.

81 Li Q, Dang CV: c-Myc overexpression uncouples DNA replication from mitosis. Mol Cell Biol 1999;19:5339–5351.
82 Gandarillas A, Davies D, Blanchard JM: Normal and c-Myc-promoted human keratinocyte differentiation both occur via a novel cell cycle involving cellular growth and endoreplication. Oncogene 2000;19:3278–3289.
83 Kuschak TI, Kuschak BC, Taylor CL, Wright JA, Wiener F, Mai S: c-Myc initiates illegitimate replication of the ribonucleotide reductase R2 gene. Oncogene 2002;21:909–920.
84 Mai S: Overexpression of c-myc precedes amplification of the gene encoding dihydrofolate reductase. Gene 1994;148:253–260.
85 Mai S, Hanley-Hyde J, Fluri M: c-Myc overexpression associated *DHFR* gene amplification in hamster, rat, mouse and human cell lines. Oncogene 1996;12:277–288.
86 Potter M: Neoplastic development in plasma cells. Immunol Rev 2003;194:177–195.
87 Taylor C, Mai S: c-Myc-associated genomic instability of the dihydrofolate reductase locus in vivo. Cancer Detect Prev 1998;22:350–356.
88 Singer MJ, Mesner LD, Friedman CL, Trask BJ, Hamlin JL: Amplification of the human dihydrofolate reductase gene via double minutes is initiated by chromosome breaks. Proc Natl Acad Sci USA 2000;97:7921–7926.
89 Kuschak TI, Taylor C, McMillan-Ward E, Israels S, Henderson DW, Mushinski JF, Wright JA, Mai S: The ribonucleotide reductase R2 gene is a non-transcribed target of c-Myc-induced genomic instability. Gene 1999;238:351–365.
90 Mai S, Hanley-Hyde J, Rainey GJ, Kuschak TI, Paul JT, Littlewood TD, Mischak H, Stevens LM, Henderson DW, Mushinski JF: Chromosomal and extrachromosomal instability of the cyclin D2 gene is induced by Myc overexpression. Neoplasia 1999;1:241–252.
91 Fukasawa K, Wiener F, Vande Woude GF, Mai S: Genomic instability and apoptosis are frequent in p53 deficient young mice. Oncogene 1997;15:1295–1302.
92 Mai S, Guffei A, Fest T, Mushinski JF: c-Myc deregulation promotes a complex network of genomic instability; in Li JJ, Li SA, Llombart-Bosch A (eds): Hormonal Carcinogenesis IV, vol 26, pp 87–97. Springer, New York, 2005.
93 Gaubatz JW: Extrachromosomal circular DNAs and genomic sequence plasticity in eukaryotic cells. Mutat Res 1990;237:271–292.
94 Mai S, Hanley-Hyde J, Coleman A, Siwarski D, Huppi K: Amplified extrachromosomal elements containing c-Myc and Pvt 1 in a mouse plasmacytoma. Genome 1995;38:780–785.
95 Von Hoff DD, McGill JR, Forseth BJ, Davidson KK, Bradley TP, Van Devanter DR, Wahl GM: Elimination of extrachromosomally amplified MYC genes from human tumor cells reduces their tumorigenicity. Proc Natl Acad Sci USA 1992;89:8165–8169.
96 Von Hoff DD, Waddelow T, Forseth B, Davidson K, Scott J, Wahl G: Hydroxyurea accelerates loss of extrachromosomally amplified genes from tumor cells. Cancer Res 1991;51:6273–6279.
97 Eckhardt SG, Dai A, Davidson KK, Forseth BJ, Wahl GM, Von Hoff DD: Induction of differentiation in HL60 cells by the reduction of extrachromosomally amplified c-*myc*. Proc Natl Acad Sci USA 1994;91:6674–6678.
98 Wahl GM: The importance of circular DNA in mammalian gene amplification. Cancer Res 1989;49:1333–1340.
99 Hahn PJ: Molecular biology of double-minute chromosomes. Bioessays 1993;15:477–484.
100 Smith G, Taylor-Kashton C, Dushnicky L, Symons S, Wright J, Mai S: c-Myc-induced Extrachromosomal Elements carry active chromatin. Neoplasia 2003;5:110–120.
101 Wiener F, Kuschak TI, Ohno S, Mai S: Deregulated expression of c-Myc in a translocation-negative plasmacytoma on extrachromosomal elements that carry IgH and myc genes. Proc Natl Acad Sci USA 1999;96:13967–13972.
102 Graux C, Cools J, Melotte C, Quentmeier H, Ferrando A, Levine R, Vermeesch JR, Stul M, Dutta B, Boeckx N, Bosly A, Heimann P, Uyttebroeck A, Mentens N, Somers R, MacLeod RA, Drexler HG, Look AT, Gilliland DG, Michaux L, Vandenberghe P, Wlodarska I, Marynen P, Hagemeijer A: Fusion of *NUP214* to *ABL1* on amplified episomes in T-cell acute lymphoblastic leukemia. Nat Genet 2004;36:1084–1089.
103 Felsher DW, Bishop JM: Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci USA 1999;96:3940–3944.

104 Mai S, Fluri M, Siwarski D, Huppi K: Genomic instability in MycER-activated Rat1A-MycER cells. Chromosome Res 1996;4:365–371.
105 Rockwood LD, Torrey TA, Kim JS, Coleman AE, Kovalchuk AL, Xiang S, Ried T, Morse HC 3rd, Janz S: Genomic instability in mouse Burkitt lymphoma is dominated by illegitimate genetic recombinations, not point mutations. Oncogene 2002;21:7235–7240.
106 Partlin MM, Homer E, Robinson H, McCormick CJ, Crouch DH, Durant ST, Matheson EC, Hall AG, Gillespie DA, Brown R: Interactions of the DNA mismatch repair proteins MLH1 and MSH2 with c-MYC and MAX. Oncogene 2003;22:819–825.
107 Davis CD, Dacquel EJ, Schut HA, Thorgeirsson SS, Snyderwine EG: In vivo mutagenicity and DNA adduct levels of heterocyclic amines in Muta mice and c-myc/lacZ double transgenic mice. Mutat Res 1996;356:287–296.
108 Vafa O, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM, Wahl GM: c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell 2002;9:1031–1044.
109 Pipiras E, Coquelle A, Bieth A, Debatisse M: Interstitial deletions and intrachromosomal amplification initiated from a double-strand break targeted to a mammalian chromosome. EMBO J 1998;17:325–333.
110 Moynahan ME, Jasin M: Loss of heterozygosity induced by a chromosomal double-strand break. Proc Natl Acad Sci USA 1997;94:8988–8993.
111 Richardson C, Jasin M: Frequent chromosomal translocations induced by DNA double-strand breaks. Nature 2000;405:697–700.
112 Land H, Parada LF, Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 1983;304:596–602.
113 Land H, Chen AC, Morgenstern JP, Parada LF, Weinberg RA: Behavior of myc and ras oncogenes in transformation of rat embryo fibroblasts. Mol Cell Biol 1986;6:1917–1925.
114 Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF: Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev 1998;12:2424–2433.
115 Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA: Creation of human tumour cells with defined genetic elements. Nature 1999;400:464–468.
116 D'Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, Moody SE, Cox JD, Ha SI, Belka GK, Golant A, Cardiff RD, Chodosh LA: c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. Nat Med 2001;7:235–239.
117 Welm AL, Kim S, Welm BE, Bishop JM: MET and MYC cooperate in mammary tumorigenesis. Proc Natl Acad Sci USA 2005;102:4324–4329.
118 Gil J, Kerai P, Lleonart M, Bernard D, Cigudosa JC, Peters G, Carnero A, Beach D: Immortalization of primary human prostate epithelial cells by c-Myc. Cancer Res 2005;65:2179–2185.
119 Boveri T: The origin of malignant tumors. Williams & Wilkins, Baltimore, 1929.
120 Marx J: Debate surges over the origins of genomic defects in cancer. Science 2002;297: 544–546.
121 Lengauer C, Kinzler KW, Vogelstein B: Genetic instabilities in human cancers. Nature 1998;396: 643–649.
122 Vogelstein B, Kinzler KW: Cancer genes and the pathways they control. Nat Med 2004;10:789–799.
123 Rajagopalan H, Lengauer C: Aneuploidy and cancer. Nature 2004;432:338–341.
124 Pihan G, Doxsey SJ: Mutations and aneuploidy: co-conspirators in cancer? Cancer Cell 2003;4: 89–94.
125 Albertson DG, Collins C, McCormick F, Gray JW: Chromosome aberrations in solid tumors. Nat Genet 2003;34:369–376.
126 Vanasse GJ, Concannon P, Willerford DM: Regulated genomic instability and neoplasia in the lymphoid lineage. Blood 1999;94:3997–4010.
127 Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM: Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci USA 1982;79:7824–7827.
128 Pear WS, Wahlstrom G, Nelson SF, Axelson H, Szeles A, Wiener F, Bazin H, Klein G, Sumegi J: 6;7 chromosomal translocation in spontaneously arising rat immunocytomas: evidence for c-myc

breakpoint clustering and correlation between isotypic expression and the c-myc target. Mol Cell Biol 1988;8:441–451.
129 Ohno S, Babonits M, Wiener F, Spira J, Klein G, Potter M: Nonrandom chromosome changes involving the Ig gene-carrying chromosomes 12 and 6 in pristane-induced mouse plasmacytomas. Cell 1979;18:1001–1007.
130 Boxer LM, Dang CV: Translocations involving c-*myc* and c-*myc* function. Oncogene 2001;20: 5595–5610.
131 Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD, Brinster RL: The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 1985;318:533–538.
132 Harris AW, Pinkert CA, Crawford M, Langdon WY, Brinster RL, Adams JM: The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. J Exp Med 1988;167:353–371.
133 Strasser A, Harris AW, Bath ML, Cory S: Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 1990;348:331–333.
134 Cheung WC, Kim JS, Linden M, Peng L, Van Ness B, Polakiewicz RD, Janz S: Novel targeted deregulation of c-Myc cooperates with Bcl-X(L) to cause plasma cell neoplasms in mice. J Clin Invest 2004;113:1763–1773.
135 Felsher DW: Reversibility of oncogene-induced cancer. Curr Opin Genet Dev 2004;14:37–42.
136 Felsher DW, Bishop JM: Reversible tumorigenesis by MYC in hematopoietic lineages. Mol Cell 1999;4:199–207.
137 Jain M, Arvanitis C, Chu K, Dewey W, Leonhardt E, Trinh M, Sundberg CD, Bishop JM, Felsher DW: Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science 2002;297:102–104.
138 Karlsson A, Deb-Basu D, Cherry A, Turner S, Ford J, Felsher DW: Defective double-strand DNA break repair and chromosomal translocations by MYC overexpression. Proc Natl Acad Sci USA 2003;100:9974–9979.
139 Karlsson A, Giuriato S, Tang F, Fung-Weier J, Levan G, Felsher DW: Genomically complex lymphomas undergo sustained tumor regression upon MYC inactivation unless they acquire novel chromosomal translocations. Blood 2003;101:2797–2803.
140 Marinkovic D, Marinkovic T, Mahr B, Hess J, Wirth T: Reversible lymphomagenesis in conditionally c-MYC expressing mice. Int J Cancer 2004;110:336–342.
141 Pelengaris S, Abouna S, Cheung L, Ifandi V, Zervou S, Khan M: Brief inactivation of c-Myc is not sufficient for sustained regression of c-Myc-induced tumours of pancreatic islets and skin epidermis. BMC Biol 2004;2:26.
142 Boxer RB, Jang JW, Sintasath L, Chodosh LA: Lack of sustained regression of c-MYC-induced mammary adenocarcinomas following brief or prolonged MYC inactivation. Cancer Cell 2004;6:577–586.
143 Jamerson MH, Johnson MD, Dickson RB: Of mice and Myc: c-Myc and mammary tumorigenesis. J Mammary Gland Biol Neoplasia 2004;9:27–37.
144 Jamerson MH, Johnson MD, Korsmeyer SJ, Furth PA, Dickson RB: Bax regulates c-Myc-induced mammary tumour apoptosis but not proliferation in MMTV-c-myc transgenic mice. Br J Cancer 2004;91:1372–1379.
145 Flores I, Murphy DJ, Swigart LB, Knies U, Evan GI: Defining the temporal requirements for Myc in the progression and maintenance of skin neoplasia. Oncogene 2004;23:5923–5930.
146 Zhu D, Qi CF, Morse HC 3rd, Janz S, Stevenson FK: Deregulated expression of the Myc cellular oncogene drives development of mouse 'Burkitt-like' lymphomas from naive B cells. Blood 2005;105:2135–2137.
147 Park SS, Kim JS, Tessarollo L, Owens JD, Peng L, Han SS, Tae Chung S, Torrey TA, Cheung WC, Polakiewicz RD, McNeil N, Ried T, Mushinski JF, Morse HC 3rd, Janz S: Insertion of c-*Myc* into *Igh* induces B-cell and plasma-cell neoplasms in mice. Cancer Res 2005;65:1306–1315.
148 Fest T, Guffei A, Williams G, Silva S, Mai S: Uncoupling of genomic instability and tumorigenesis in a mouse model of Burkitt's lymphoma expressing a conditional box II-deleted Myc protein. Oncogene 2005;24:2944–2953.
149 Fest T, Mougey V, Dalstein V, Hagerty M, Milette D, Silva S, Mai S: c-MYC overexpression in Ba/F3 cells simultaneously elicits genomic instability and apoptosis. Oncogene 2002;21:2981–2990.

150 Nesbit CE, Tersak JM, Prochownik EV: *MYC* oncogenes and human neoplastic disease. Oncogene 1999;18:3004–3016.
151 Herrick J, Conti C, Teissier S, Thierry F, Couturier J, Sastre-Garau X, Favre M, Orth G, Bensimon A: Genomic organization of amplified *MYC* genes suggests distinct mechanisms of amplification in tumorigenesis. Cancer Res 2005;65:1174–1179.
152 Squire JA, Pei J, Marrano P, Beheshti B, Bayani J, Lim G, Moldovan L, Zielenska M: High-resolution mapping of amplifications and deletions in pediatric osteosarcoma by use of CGH analysis of cDNA microarrays. Genes Chromosomes Cancer 2003;38:215–225.
153 Lim G, Karaskova J, Beheshti B, Vukovic B, Bayani J, Selvarajah S, Watson SK, Lam WL, Zielenska M, Squire JA: An integrated mBAND and submegabase resolution tiling set (SMRT) CGH array analysis of focal amplification, microdeletions, and ladder structures consistent with breakage-fusion-bridge cycle events in osteosarcoma. Genes Chromosomes Cancer 2005;42:392–403.
154 Refaeli Y, Field KA, Turner BC, Trumpp A, Bishop JM: The protooncogene *MYC* can break B cell tolerance. Proc Natl Acad Sci USA 2005;102:4097–4102.
155 Pelengaris S, Khan M, Evan GI: Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. Cell 2002;109:321–334.
156 Laybutt DR, Weir GC, Kaneto H, Lebet J, Palmiter RD, Sharma A, Bonner-Weir S: Overexpression of c-Myc in beta-cells of transgenic mice causes proliferation and apoptosis, downregulation of insulin gene expression, and diabetes. Diabetes 2002;51:1793–1804.
157 Rothermund K, Rogulski K, Fernandes E, Whiting A, Sedivy J, Pu L, Prochownik EV: c-Myc-independent restoration of multiple phenotypes by two c-Myc target genes with overlapping functions. Cancer Res 2005;65:2097–2107.

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Nijmegen Breakage Syndrome and Functions of the Responsible Protein, NBS1

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Abstract

Nijmegen breakage syndrome (NBS) is a rare recessive genetic disorder, characterized by bird-like facial appearance, early growth retardation, congenital microcephaly, immunodeficiency and high frequency of malignancies. NBS belongs to the so-called chromosome instability syndromes; in fact, NBS cells display spontaneous chromosomal aberrations and are hypersensitive to DNA double-strand break-inducing agents, such as ionizing radiations. *NBS1*, the gene underlying the disease, is located on human chromosome 8q21. The disease appears to be prevalent in the Eastern and Central European population where more than 90% of patients are homozygous for the founder mutation 657del5 leading to a truncated variant of the protein. NBS1 forms a multimeric complex with MRE11/RAD50 nuclease at the C-terminus and retains or recruits them at the vicinity of sites of DNA damage by direct binding to histone H2AX, which is phosphorylated by PI3-kinase family, such as ATM, in response to DNA damage. Thereafter, the NBS1-complex proceeds to rejoin double-strand breaks predominantly by homologous recombination repair in vertebrates. NBS cells also show to be defective in the activation of intra-S phase checkpoint. We review here some cellular and molecular aspects of NBS, which might contribute to the clinical symptoms of the disease.

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Clinical Symptoms

In 1981, Weemaes was the first to describe two Dutch brothers affected with a rare recessive genetic disorder, named Nijmegen breakage syndrome

(NBS) [1]. After the first recognition of the disorder, the disease appeared to be prevalent in the Eastern and Central European population, particularly in Poland and Czech Republic. It is assumed that the Dutch patients may have had Bohemian ancestors who immigrated to Holland in the first half of the 17th century. So far about 100 patients have been included in the international Nijmegen breakage syndrome registry. The frequency of heterozygous carriers in the Eastern and Central European population is ranging from 1:130 to 1:158 [2].

NBS patients display several characteristic facial features; namely, all patients have a typical bird-like face, characterized by a receding forehead, a prominent mid-face with a long nose and a long philtrum, and a receding mandible. Most patients also show epicanthal folds, large ears, and sparse hair. Severe and progressive microcephaly after the first month of life has been detected in 100% of NBS patients as recently reported [3]. Growth retardation is seen as a cardinal symptom of the disease, and short stature usually becomes apparent by 2 years of age. In about half of the patients borderline to mild mental retardation has been reported; however, in those patients with normal IQ during early childhood, progressive retardation occurs as they grow older.

Café au lait spots and sun sensitivity of the eyelids have also been reported, whereas cutaneous telangiectasia is only occasionally seen. Minor malformations, such as clinodactyly and syndactyly are present in about half of the patients, while less common malformations are anal atresia, hydronephrosis, and hip dysplasia. Clinical aspects related to infertility, as ovarian dysgenesis with primary amenorrhea and elevated gonadotrophin levels, have been described in several NBS patients [4].

NBS patients are particularly prone to recurrent infections, such as those of the respiratory and urinary tract, which in some cases have fatal outcome. Immunological disturbances in humoral immunity observed in NBS are agammaglobulinaemia (in about one third of NBS), IgG and IgA deficiency, as well as IgG2 and IgG4 deficiency. 10% of patients show a normal Ig status. Cellular immunity is also consistently compromised in NBS, whose patients showed reduced percentages of total CD3+ cells and CD4+ cells and a decreased CD4+/CD8+ ratio [4]. The response of lymphocytes to phytohemagglutinin (PHA) was decreased in nearly all NBS patients.

Patients also have a very high risk for developing cancer. So far, 22 of 55 INR patients (age range from 1 to 22 years) have developed a malignancy. Of these, 16 patients developed lymphomas and the remaining six have leukemia, glioma, medulloblastoma, or rhabdomyosarcoma [5]. The majority of lymphomas are non-Hodgkin's lymphomas (NHL), particularly diffuse large B-cell lymphomas (DLBL), which are rarely observed in childhood. Similarly, peripheral T-cell lymphomas, unusual in childhood, are often found in NBS patients. In addition, recent reports suggest a strong correlation between NBS and

perineal region rhabdomyosarcoma [6] and medulloblastoma [7] implying that mutations in the *NBS1* gene predispose to a variety of childhood malignancies. Moreover, a high frequency of heterozygous mutations in the *NBS1* gene has been reported in otherwise healthy children who developed acute lymphoblastic leukemia [8]. Similar findings have later been described by several researches [9–12] who assessed the frequency of the 657del5 mutation in different cohorts of patients affected with specific types of tumors. Steffen and coworkers [13] evaluated the frequency of the 657del5 mutation and of the R215W molecular variant of the *NBS1* gene in a large group of cancer patients. All these studies confirm that heterozygous carriers of *NBS1* mutations have an enhanced risk to develop malignant tumors, particularly melanoma, breast cancer, prostate cancer, colorectal cancers, and possibly also lymphoid malignancies.

Interestingly, NBS shows overlapping clinical and cellular features with a number of others hereditary disorders associated with impaired DNA damage response mechanisms (table 1). Therefore, based on common clinical signs and cellular characteristics similarities, NBS was initially thought to be a clinical variant of AT. However, the detailed clinical features are quite different, since patients with NBS do not show elevation of α-fetoprotein, cerebellar ataxia, or telangiectasia but do have microcephaly and growth retardation that might be explained by the involvement of NBS1 in the ATR-signaling pathway (see below).

Chromosomal Instability and Radiosensitivity of NBS Cells

Although karyotypes are basically normal, 10–45% of metaphases from cultured mitogen-stimulated T cells from NBS patients show chromosome instability, such as spontaneous chromosomal breaks, gaps, or rearrangements. Most of the rearrangements preferentially involve chromosomes 7 and 14, with breakpoints at the sites of IgH or TCR genes. Chromosome aberrations include inv(7)(p13q35), t(7;14)(p13;q11), t(7;14)(q35;q11), t(7;7)(p13;q35), and t(14;14)(p11;q32) [14, 15]. Primary fibroblast cells from NBS patients display poor growth in culture and chromosome analysis also shows spontaneous chromosome instability, such as gaps and breaks, most of which are chromatid-type not occurring at preferential sites. End-to-end telomeric fusions are frequently detected.

A relevant aspect of NBS is represented by its sensitivity to ionizing radiation both in patients and cultured cells [16]. NBS cells display sensitivity toward agents that are able to induce DNA double-strand breaks (DSBs) through direct action, or indirectly through replication fork stall. In fact, high sensitivity to the radiomimetic chemicals bleomycin and streptonigrin as well as inhibitors of topoisomerases I and II, camptothecin and etoposide, has been reported [4]. NBS

Table 1. List of disorders associated with a genomic instability and sharing features with those observed in NBS

Clinical symptoms	Syndrome (OMIM)[a]					
	NBS (251260)	AT (208900)	ATLD (604391)	FA (227650)	LIG4 (606593)	ATR-Seckel (210600)
Microcephaly	+	–	–	+	+	+
Dysmorphic facial features	+	–	–	–	+	+
Growth delay	+	–	–	+	+	+
Ataxia	–	+	+	–	–	–
Telangiectasia	–	+	–	–	+	–
Increased α-fetoprotein	–	+	–	+	–	–
Immunodeficiency	+	+	–	*	+	–
Cancer predisposition	+	+	NK	+	1/5 patients	NK
7/14 chromosome translocations	+	+	+	–	–	–
Radiation sensitivity	+	+	+	FA-D2	+	–
Underlying gene(s)	*NBS1*	*ATM*	*MRE11*	*FANCA, FANCB, FANCC, BRCA2, FANCD2, FANCE, FANCF, FANCL*	*LIG4*	*ATR*

[a] +: Presence of feature; –: absence; NK: not known; *: severe pancytopenia due to bone marrow failure.

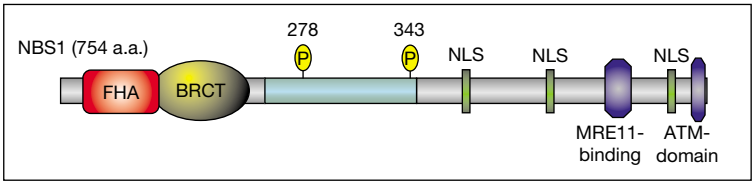

Fig. 1. Structure of NBS1 protein. NBS1 consists of three functional regions: FHA/BRCT domain at the N-terminus, ATM-phosphorylated serine residues at a central region, MRE11- and ATM-binding regions at the C-terminus.

seems to be also sensitive to the cross-linking agent mitomycin C. Treatments of NBS cells with DSB-inducing agents described above are reflected by an at least two-fold increase in cell death, compared to normal cells [17]. Such sensitivity is also observed by increased frequency of chromosomal aberrations, mainly containing the chromatid-type [16, 18]. Because of the induced and spontaneous chromosome aberrations NBS has been categorized with so-called chromosome instability disorders [19].

NBS1 Gene and Protein Structures of NBS1/MRE11/RAD50

The *NBS1* gene was mapped to 8q21-q24 by functional complementation assays [20, 21] and by a familial linkage analysis [22, 23] and from this candidate region the *NBS1* gene was identified [24–26]. The *NBS1* gene consists of 16 exons, is 50 kb in size, and encodes a 754-amino acid NBS1 protein. A 5-bp *NBS1* deletion, 657del5, has been found in most NBS families (more than 90%) with Slavic origins prevalently in the Eastern and Central European population, demonstrating the presence of a common founder effect. All nine mutations presently reported are located between nucleotides 657 and 1142, and are believed to be hypomorphic mutations, since the synthesis of a variant form of NBS1 protein, with a lower molecular weight of 70 kDa, has been demonstrated in NBS cells carrying the classical 657del5 mutation [27]. The 835del4 and the 900del25 deletions also produce variant proteins of 60 and 55 kDa, respectively [28].

The NBS1 protein contains four functional regions: the N-terminus (1–196 a.a.), a central region (278–343 a.a.), a C-terminus (665–693 a.a.) (fig. 1) and an extreme C-terminus portion (734–754 a.a.). Weak (29%) homology to yeast Xrs2 protein was first recognized at the N-terminal sequence. This region

includes a fork head-associated (FHA) domain (24–108 a.a.) and a BRCA1 C-terminus (BRCT) domain (108–196 a.a.). FHA and BRCT domains are widely conserved in eukaryotic nuclear proteins that are related to cell-cycle checkpoints or DNA repair. The BRCT domain has not been identified in budding yeast but is conserved in fission yeast with 18% identity. However, Nbs1 homologs in the fungus *A. nidulan*s possess neither a FHA nor a BRCT domain [29]. We previously demonstrated that the FHA/BRCT domain directly binds to phosphorylated histone γ-H2AX and retains or recruits the MRE11/RAD50 complex to the vicinity of sites of DSBs [30].

NBS1 protein is a component of the MRN complex that contains MRE11 and RAD50 proteins. The C-terminal region of NBS1 binds to the MR complex. This region is well conserved throughout eukaryotes, including *A. nidulans*. Yeast two-hybrid experiments showed that this region at the C-terminus binds to MRE11 and is essential for nuclear transportation of the MR complex. Very recently, in addition to the previously characterized regions of NBS1, a conserved motif of 20 residues at its extreme C-terminus has been shown to interact with ATM, which activates the ATM-dependent phosphorylation of various substrates [31, 32].

The central region of NBS1 functions in signal transduction for damage response. The serine residues at positions 278 and 343 are phosphorylated by ATM in response to radiation both in vitro and in vivo, and these phosphorylations are associated with the role for intra-S cell cycle checkpoint [33–36]. Therefore, these serine residues are well conserved in vertebrates [37], although serine 278 is substituted by threonine in mouse Nbs1 and no phosphorylation sites have been identified in budding yeast, fission yeast, and *A. nidulans.*

Cell Cycle Checkpoint Defect in NBS

The well-characterized cell cycle control defect in NBS cells is the radiation resistant DNA synthesis (RDS), in which NBS cells continue DNA synthesis in the presence of radiation-induced DNA damage. RDS is due to a failure of the intra-S cell cycle checkpoint. Cells from patients with AT-like disease (ATLD), which are mutated in MRE11, also display pronounced RDS, and hence the MRN complex could be involved in the S-phase transition. This hypothesis is supported by fission yeast experiments demonstrating that Rad32/Rad50/Nbs1 (the homolog of human MRN complex) acts specifically in the intra-S cell cycle checkpoint [38].

It has been reported that at least three parallel pathways participate in the intra-S cell cycle checkpoint: the NBS1-dependent ATM/NBS1/SMC1 pathway

[39, 40], the NBS1-independent ATM/Chk2/Cdc25A/Cdk2 pathway [41], and the NBS1-dependent ATM/FANCD2 pathway [42]. In the first pathway, ATM phosphorylates Ser-957 and Ser-966 of SMC1, a component of the cohesin complex, which is involved in the activation of the intra-S cell cycle checkpoint. SMC1 phosphorylation requires the phosphorylation of NBS1 on both Ser-278 and Ser-343. In the second pathway, ATM activates CHK2, which phosphorylates the cell cycle regulator CDC25A, leading to its degradation through the poly-ubiquitination-mediated proteolysis pathway. It is likely that phosphorylation of CHK2 and CDC25A is normal in NBS cells, while several reports indicate the requirement of NBS1 at low damage levels. Due to the presence of this NBS1-independent pathway, the level of RDS in NBS cells might fall between that of AT and wild-type control cells [41]. Recently, it was reported that ATM phosphorylates Ser-222 of FANCD2 (mutated in the chromosomal instability syndrome Fanconi's anemia) and this event also influences the intra-S cell cycle checkpoint [42]. This FANCD2 phosphorylation requires Ser-343 phosphorylation of NBS1 by an ATM-dependent mechanism [43].

FHA/BRCT-deleted cells abrogate intra-S cell cycle checkpoint and are defective in phosphorylation of Ser-343, while phosphorylation of Ser-278 is normal [44]. Consistent with this, an NBS1 S343A mutant fails to restore S-phase checkpoint, indicating that this checkpoint requires the phosphorylation of Ser-343 and the FHA/BRCT domain. On the other hand, FHA/BRCT mutants are able to restore the downstream phosphorylation of CHK2 and SMC1, which is defective in NBS cells [38, 45, 46]. This observation may be well related to the presence of an intact extreme C-terminus of NBS1 in FHA/BRCT mutants. In fact, deletion of the C-terminus leads to a strongly reduced or completely impaired phosphorylation of SMC1, CHK2 and NBS1 Ser-343 [31]. However, FHA/BRCT-deleted cells, similar to those ablated in the ATM-NBS1 interaction, show RDS and this domain is required for checkpoint activation, as well as in chromosomal radiosensitivity, by physical interaction between MRN complex and SMC1 (Antoccia et al., unpublished results). In the light of the recent discovery of the ATM-NBS1 interaction domain, the roles of both the FHA/BRCT domain and the MRE11-binding region in intra-S cell cycle checkpoint [45] remain to be re-evaluated.

Beside the intra-S phase checkpoint defect, some authors showed that NBS1 could be involved in G1 and G2 checkpoints in vertebrates. However, although the impairments of CHK2 phosphorylation and G2 checkpoint are observed in NBS cells [47], and are strictly dependent on the ATM-NBS1 interaction domain, other researchers report proficient G2 checkpoint activation [48, 49]. Similar to CHK2, damage-induced cellular levels of p53 and $p21^{WAF1/CIP1}$ have been reported to be sub-optimal in NBS cells compared to cells from unaffected individuals [18, 50, 51]. Thus, defects of G1 and G2 checkpoints in NBS

cells are partial in nature, as is the case for the intra-S cell cycle checkpoint, and the role of NBS1 in G1 and G2 checkpoint integrity remains controversial.

The Role of NBS1 in Immunodeficiency

It is known that during immune development many of the damage response mechanisms that evolved to maintain genomic stability are exploited to generate genetic diversity in developing T and B cells. Three defined processes contribute to the diversity of immunological components: V(D)J recombination, class switch recombination (CSR) and somatic hypermutation. As a consequence of the overlap between genomic stability and genetic diversity, it is now clear that genetic diseases associated with an impaired DNA damage response are also defective in the immune response. In fact, during processes used to create immunoglobulin diversity, namely V(D)J recombination and CSR, DSBs are introduced into DNA, thus it is not surprising that disorders associated with defects in the response to DSBs display immunodeficiency [52]. In this context, V(D)J recombination was measured in NBS cells by several groups and was shown to be normal [53, 54].

On the other hand, the humoral immune deficiency of IgG and IgA with normal IgM levels, as observed in NBS patients, seems to suggest that CSR in B-cells might be impaired. In addition, it has been shown that during CSR occurring in G1 phase, DSBs introduced in the S regions are associated with H2AX phosphorylation and the MRN complex is recruited to the IgH locus [55]. Furthermore, structural and biochemical studies have shown that the MRN complex tethers DNA ends, and stimulates intermolecular joining by non-homologous recombination [56]. Experimental evidence has been accumulated in in vitro systems showing that the MRN complex plays a role in CSR [57, 58].

However, since null mutations for *NBS1*, similar to *RAD50* and *MRE11*, cause early embryonic lethality in mouse models [59], it is difficult to assess the exact role of Nbs1 in in vivo systems. As well as lethality, the hypomorphic nature of NBS1 hampers to define the role of NBS1. To overcome this limitation, murine models have recently been established by a Cre-loxP-mediated recombination, which allows conditional inactivation of Nbs1 in B lymphocytes [60, 61]. It was shown that irradiated B cells display radioresistant DNA synthesis and an enhanced chromosomal sensitivity to irradiation. In addition, spontaneous structural and numerical chromosomal alterations were detected at a high rate in these mice, indicating that physiological levels of Nbs1 are required to maintain the integrity of chromosome structure and number. More interestingly, the authors reported $Nbs1^{\Delta/-}$ conditional mutant mice showing

immunological defects. Serum Ig levels were measured in these mice to determine whether Nbs1 deficiency affects CSR. Though the IgM serum levels were similar in $Nbs1^{\Delta/-}$ and control mice, IgG, IgG1, IgG2b and IgG3 were reduced in $Nbs1^{\Delta/-}$ mice. The conditional inactivation of Nbs1 in B cells reduces the frequencies of switched cells to more than 50%. The observation that CSR is reduced in $Nbs1^{\Delta/-}$ mice to a similar extent to that observed in $ATM^{-/-}$ mice has led to the suggestion that, in addition to tethering DNA ends during CSR, Nbs1 may regulate accumulation of ATM at S-region breaks and the phosphorylation of substrates required for CSR. In addition to the role of Nbs1 in CSR, it is also relevant for the survival of B lymphocytes. In conclusion, Nbs1 plays an important role in the rejoining of class-switch recombination breaks which may be related to the preponderance of B cell lymphoma among NBS patients.

NBS1 is Required for Recruitment of ATM to Sites of DNA Damage and for ATM- and ATR-Dependent Phosphorylation Events

The aim of the DNA damage response pathways is to translate the signal originating from a DNA lesion into cellular responses, such as cell cycle checkpoint activation and DNA repair. To achieve this, three major groups of proteins act together: sensor proteins that are responsible for DNA damage recognition, transducer proteins that amplify the signal and effector proteins that control cell cycle and repair.

The first step, which allows NBS1 to recognize DSBs and retains or recruits the MRN complex to the damaged site shortly after their generation, is not completely understood. We proposed a two-step binding model of NBS1 for recognition and rejoining of DNA damage, in which NBS1 retains or recruits MRE11/RAD50 nuclease at the vicinity of sites of DNA damage by direct binding to histone H2AX, and thereafter, proceeds to rejoin double-strand breaks predominantly by homologous recombination repair [62]. However, the hierarchical association of ATM and NBS1 has been rather elusive. ATM and ATR protein kinase are rapidly activated in response to DNA damage and act as transducer proteins. In particular, ATM responds mainly to DSBs, whereas ATR is activated in response to single stranded DNA and stalled replication forks [63]. Since ATM phosphorylates NBS1 in response to DSB-inducing agents [33–36], ATM must function upstream of, and prior to, NBS1. But, this phosphorylation of NBS1 is not required for NBS1 recruitment to damaged sites, as shown by the proficient radiation-induced foci formation in NBS1 S343A cells [30]. Similarly, early MRE11 foci are formed by

a mechanism independent of ATM, ATR and DNA-PKcs, suggesting that MRE11 acts as a sensor in that it is the first protein to recognize DSBs [64]. This ATM-independent NBS1 foci formation is observed at telomere ends, to which NBS1 is recruited by binding to MRE11 via the C-terminus of NBS1 (unpublished observations). This should also be the case for DNA damage responses, since Xrs2 is recruited to DSBs through a mechanism independent of Tel1, a yeast homolog of ATM that is similarly dependent on the C-terminus of Xrs2 [65]. As stated before, a conserved motif of 20 residues at its extreme C-terminus has been shown to be sufficient for the interaction with ATM [44]. The observation that an NBS1 mutant impaired in MRE11 binding still interacts with ATM, clearly indicates that ATM-NBS1 interaction is independent of an intact MRN complex. Interestingly, regions with sequence homology to the NBS1 C-terminus have been also found in the C-terminal regions of human ATRIP and Ku80 proteins that are required for their interaction with ATR and DNA-PKcs, respectively.

ATM activation requires its autophosphorylation on Ser1981, and then this autophosphorylated species co-localizes with the radiation-induced foci of γ-H2AX, the phosphorylated form of histone H2AX [66]. By contrast, no foci of phospho-Ser1981-ATM were found in cells complemented with the truncated ATM-NBS1 interaction domain. In this case, the pan-nuclear staining of phospho-Ser1981-ATM was observed despite the presence of γ-H2AX and MRE11 foci. Hence, the ATM interaction domain at NBS1 C-terminus is required to recruit ATM to sites of DNA damage. Since it is known that the MRN complex facilitates ATM-dependent signalling and stimulates the kinase activity of ATM in vitro toward several substrates [67–69], it has been tested whether ablation of the ATM-NBS1 interaction affected phosphorylation of specific substrates. The ablation of the NBS1-ATM interaction selectively led to a diminished phosphorylation of CHK2 and SMC1, whereas neither ATM autophosphorylation was affected nor phosphorylation of Ser-15 of p53 impaired [31, 33]. H2AX phosphorylation occurred independently from the NBS1 status.

Recent findings suggest that NBS1 functions also in the ATR-signalling pathway. NBS1 co-localizes with RPA (Replication Protein A), which in turn co-localizes with ATR in the early step of damage response of HU-induced replication stalled lesions [70]. However, while RPA recruitment to damage sites occurs without NBS1, recruitment or retention of ATR is strongly decreased after replication stalling in NBS cells. The role of NBS1 in the ATR-pathway is similar to its role in the ATM-signalling, in which NBS1 is required for ATR-dependent phosphorylation of several substrates in response to replication stall introduced by UV and HU [70]. In fact, NBS cells show similar defects as ATR-Seckel syndrome cells in ATR-dependent phosphorylation of p53, Chk1 and c-jun after treatment. Restart of DNA synthesis at stalled replication forks is

also compromised in NBS as well as in ATR cells. An additional interesting aspect is represented by the impairment of NBS cells in ubiquitination of FANCD2 after HU exposure, a pathway which is ATR-dependent.

In conclusion, recent data strongly suggest that NBS1 plays an important role not only in the ATM-dependent signalling pathway, but also in the ATR-dependent one. It has been proposed that NBS features represent the result of these combined defects (see table 1). In this respect, NBS shares some clinical features with AT (immunodeficiency and predisposition to malignancies) and ATR-Seckel syndrome (microcephaly, growth delay, facial signs). In addition, it has been shown that NBS1 clinically overlaps with FA (Fanconi anemia). This overlapping may be explained by taking into account that ATR is required for FANCD2 ubiquitination, and this event in turn is required for FA activation, coupling ATR signalling to the activation of the FA pathway [71, 72]. The observation on NBS1 requirement for FANCD2 ubiquitination after induction of stalled replication forks suggests a role of NBS1 also in the FA pathway. In agreement with an involvement of NBS1 in FA pathway, it has been shown that NBS1 cells are sensitive to the cross-linking agents, which was considered as a cellular hallmark of FA [17]. In addition, several patients originally diagnosed as FA, have been instead proved to be NBS after further molecular analysis [42, 73].

References

1 Weemaes CM, Hustinx TW, Scheres JM, van Munster PJ, Bakkeren JA, Taalman RD: A new chromosomal instability disorder: the Nijmegen breakage syndrome. Acta Pediatr Scand 1981;70:557–564.

2 Varon R, Seemanova E, Chrzanowska K, Hnateyko O, Piekutowska-Abramczuk D, Krajewska-Walasek M, Sykut-Cegielska J, Sperling K, Reis A: Clinical ascertainment of Nijmegen breakage syndrome (NBS) and prevalence of the major mutation, 657del5, in three Slav populations. Eur J Hum Genet 2000;8:900–902.

3 Seeman P, Gebertova K, Paderova K, Sperling K, Seemanova E: Nijmegen breakage syndrome in 13% of age-matched Czech children with primary microcephaly. Pediatr Neurol 2004;30:195–200.

4 Digweed M, Sperling K: Nijmegen breakage syndrome: clinical manifestation of defective response to DNA double-strand breaks. DNA Repair 2004;3:1207–1217.

5 The International Nijmegen Breakage Syndrome Study Group: Nijmegen Breakage Syndrome. Arch Dis Child 2000;82:400–406.

6 Meyer S, Kingston H, Taylor ARM, Byrd PJ, Last JI, Brennan BM, Trueman S, Kelsey A, Taylor GM, Eden OB: Rhabdomyosarcoma in Nijmegen breakage syndrome: strong association with perianal primary site. Cancer Genet Cytogenet 2004;154:169–174.

7 Distel L, Neubauer S, Varon R, Holter W, Grabenbauer G: Fatal toxicity following radio- and chemotherapy of medulloblastoma in a child with unrecognized Nijmegen breakage syndrome. Med Pediatr Oncol 2003;41:44–48.

8 Varon R, Reis A, Henze G, von Einsiedel HG, Sperling K, Seeger K: Mutations in the Nijmegen Breakage Syndrome gene (*NBS1*) in childhood acute lymphoblastic leukemia (ALL). Cancer Res 2001;61:3570–3572.

9 Debniak T, Gorski B, Cybulski C, Jakubowska A, Kurzawski G, Lener M, Mierzejewski M, Masojc B, Medrek K, Kladny J, Zaluga E, Maleszka R, Chosia M, Lubinski J: Germline 657del5 mutation in the *NBS1* gene in patients with malignant melanoma of the skin. Melanoma Res 2003;13:365–370.
10 Gorski B, Debniak T, Masojc B, Mierzejewski M, Medrek K, Cybulski C, Jakubowska A, Kurzawski G, Chosia M, Scott R, Lubinski J: Germline 657del5 mutation in the *NBS1* gene in breast cancer patients. Int J Cancer 2003;106:379–381.
11 Resnick IB, Kondratenko I, Pashanov E, Maschan AA, Karachunsky A, Togoev O, Timakov A, Polyakov A, Tverskaya S, Evgrafov O, Roumiantsev AG: 657del5 mutation in the gene for Nijmegen breakage syndrome (*NBS1*) in a cohort of Russian children with lymphoid tissue malignancies and controls. Am J Med Genet 2003;120:174–179.
12 Cybulski C, Gorski B, Debniak T, Gliniewicz B, Mierzejewski M, Masojc B, Jakubowska A, Matyjasik J, Zlowocka E, Sikorski A, Narod SA, Lubinski J: *NBS1* is a prostate cancer susceptibility gene. Cancer Res 2004;64:1215–1219.
13 Steffen J, Varon R, Mosor M, Maneva G, Maurer M, Stumm M, Nowakowska D, Rubach M, Kosakowska E, Ruka W, Nowecki Z, Rutkowski P, Demkow T, Sadowska M, Bidzinski M, Gawrychowski K, Sperling K: Increased cancer risk of heterozygotes with *NBS1* germline mutations in Poland. Int J Cancer 2004;111:67–71.
14 van der Burgt I, Chrzanowska KH, Smeets D, Weemaes C: Nijmegen breakage syndrome. J Med Genet 1996;33:153–156.
15 Chrzanowska KH, Kleijer WJ, Krajewska-Walesk M, Bialecka M, Gutkowska A, Goyrluk-Kozakiewicz B, Michalkiewicz J, Stachowscki J, Gregorek H, Lyson-Wojciechowska G, et al: Eleven Polish patients with microcephaly, immunodeficiency, and chromosomal instability: the Nijmegen breakage syndrome. Am J Med Genet 1995;57:462–471.
16 Taalman RD, Jaspers NG, Scheres JM, de Wit J, Hustinx TW: Hypersensitivity to ionizing radiation, in vitro, a new chromosomal breakage disorder, the Nijmegen breakage syndrome. Mutat Res 1983;112:23–32.
17 Kraakman-van der Zwet M, Overkamp WJ, Friedl AA, Klein B, Verhaegh GW, Jaspers NG, Midro AT, Eckardt-Schupp F, Lohman PH, Zdzienicka MZ: Immortalization and characterization of Nijmegen breakage syndrome fibroblasts. Mutat Res 1999;434:17–27.
18 Antoccia A, Stumm M, Saar K, Ricordy R, Maraschio P, Tanzarella C: Impaired p53-mediated DNA damage response, cell-cycle disturbance and chromosome aberrations in Nijmegen breakage syndrome lymphoblastoid cell lines. Int J Radiat Biol 1999;75:583–591.
19 Digweed M: Response to environmental carcinogens in DNA-repair-deficient disorders. Toxicology 2003;193:111–124.
20 Komatsu K, Matsuura S, Tauchi H, Endo S, Kodama S, Smeets D, Weemaes C, Oshimura M: The gene for Nijmegen breakage syndrome (V2) is not located on chromosome 11. Am J Hum Genet 1996;58:885–888.
21 Matsuura S, Weemaes C, Smeets D, Takami H, Kondo N, Sakamoto S, Yano N, Nakamura A, Tauchi H, Endo S, Komatsu K: Genetic mapping using microcell-mediated chromosome transfer suggests a locus for Nijmegen breakage syndrome at chromosome 8q21-24. Am J Hum Genet 1997;60:1487–1494.
22 Saar K, Chrzanowska KH, Stumm M, Jung M, Nurnberg G, Wienker TF, Seemanova E, Wegner RD, Reis A, Sperling K: The gene for the ataxia-telangiectasia variant, Nijmegen breakage syndrome, maps to a 1-cM interval on chromosome 8q21. Am J Hum Genet 1997;60:605–610.
23 Cerosaletti KM, Lange E, Strinhman HM, Weemaes C, Smeets D, Solder B, Belohradsky BH, Taylor AMR, Karnes P, Elloptt A, Komatsu K, Gatti RA, Boehnke M, Concannon P: Fine localization of the Nijmegen breakage syndrome gene to 8q21: evidence for a common founder haplotype. Am J Hum Genet 1998;63:125–134.
24 Matsuura S, Tauchi H, Nakamura A, Kondo N, Sakamoto S, Endo S, Smeets D, Solder B, Belohradsky BH, Der Kaloustian VM, Oshimura M, Isomura M, Nakamura Y, Komatsu K: Positional cloning of the gene for Nijmegen breakage syndrome. Nat Genet 1998;19:179–181.
25 Carney JP, Maser RS, Olivares H, Davis EM, Le Beau M, Yates JR 3rd, Hays L, Morgan WF, Petrini JH: The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell 1998;93:477–486.

26 Varon R, Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH, Saar K, Beckmann G, Seemanova E, Cooper PR, Nowak NJ, Stumm M, Weemaes C, Gatti RA, Wilson RK, Digweed M, Rosenthal A, Sperling K, Concannon P, Reis A: Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. Cell 1998;93:467–476.
27 Maser RS, Zinkel R, Petrini JH: An alternative mode of translation permits production of a variant NBS1 protein from the common Nijmegen breakage syndrome allele. Nat Genet 2001;27: 417–421.
28 Tanzarella C, Antoccia A, Spadoni E, di Masi A, Pecile V, Demori E, Varon R, Marseglia GL, Tiepolo L, Maraschio P: Chromosome instability and nibrin protein variants in NBS heterozygotes. Eur J Hum Genet 2003;11:297–303.
29 Ueno M, Nakazaki T, Akamatsu Y, Watanabe K, Tomita K, Lindsay HD, Shinagawa H, Iwasaki H: Molecular characterization of the *Schizosaccharomyces pombe* Nbs1 gene involved in DNA repair and telomere maintenance. Mol Cell Biol 2003;23:6553–6563.
30 Kobayashi J, Tauchi H, Sakamoto S, Nakamura A, Morishima K, Matsuura S, Kobayashi T, Tamai K, Tanimoto K, Komatsu K: NBS1 localizes to gamma-H2AX foci through interaction with the FHA/BRCT domain. Curr Biol 2002;12:1846–1851.
31 Falck J, Coates J, Jackson SP: Conserved modes of recruitment of ATM, ATR and DNA PKcs to sites of DNA damage. Nature 2005;434:605–611.
32 Lee JH, Paull TT: ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science 2005;308:551–554.
33 Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JHJ, Kastan MB: ATM phosphorylates p95/NBS1 in a S-phase checkpoint pathway. Nature 2000;404:613–617.
34 Zhao S, Weng YC, Yuan SS, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY: Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. Nature 2000;405:473–477.
35 Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB, Crick KE, Pierce KA, Lane WS, Rathbun G, Livingston DM, Weaver DT: ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. Nature 2000;405:477–482.
36 Gatei M, Young D, Cerosaletti KM, Desai-Mehta A, Spring K, Kozlov S, Lavin MF, Gatti RA, Concannon P, Khanna K: ATM-dependent phosphorylation of nibrin in response to radiation exposure. Nat Genet 2000;25:115–119.
37 Tauchi H, Kobayashi J, Morishima K, Matsuura S, Nakamura A, Shiraishi T, Ito E, Masnada D, Delia D, Komatsu K: The forkhead-associated domain of NBS1 is essential for nuclear foci formation after irradiation but not essential for hRAD50/hMRE11/NBS1 complex DNA repair activity. J Biol Chem 2001;276:12–15.
38 Chahwan C, Nakamura TM, Sivakumar S, Russell P, Rhind N: The fission yeast Rad32 (Mre11)-Rad50-Nbs1 complex is required for the S-phase DNA damage checkpoint. Mol Cell Biol 2003;23:6564–6573.
39 Kim ST, Xu B, Kastan MB: Involvement of the cohesion protein, Smc1, in Atm-dependent and independent responses to DNA damage. Genes Dev 2002;16:560–570.
40 Yazdi PT, Wang Y, Zhao S, Patel N, Lee EY, Qin J: SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. Genes Dev 2002;16:571–582.
41 Falck J, Petrini JHJ, Williams BR, Lukas J, Bartek J: The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. Nat Genet 2002;30:290–294.
42 Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, Kim ST, Lane WS, Kastan MB, D'Andrea AD: Convergence of the Fanconi anemia and ataxia telangiectasia signaling pathways. Cell 2002;109:459–472.
43 Nakanishi K, Taniguchi T, Ranganathanan V, New HV, Moreau LA, Stostsky M, Mathew CG, Kastan MB, Weaver DT, D'Andrea A: Interaction of FANCD2 and NBS1 in DNA damage response. Nat Cell Biol 2002;4:913–920.
44 Zhao S, Renthal W, Lee EY: Functional analysis of FHA and BRCT domains of NBS1 in chromatin association and DNA damage responses. Nucleic Acids Res 2002;30:4815–4822.
45 Lee JH, Xu B, Lee CH, Ahn JY, Song MS, Lee H, Canman CE, Lee JS, Kastan MB, Lim DS: Distinct functions of Nijmegen breakage syndrome in Ataxia Telangiectasia mutated-dependent responses to DNA damage. Mol Cancer Res 2003;1:674–681.

46 Cerosaletti KM, Concannon P: Nibrin forkhead-associated domain and breast cancer C-terminal domain are both required for nuclear focus formation and phosphorylation. J Biol Chem 2003;278: 21944–21951.
47 Buscemi G, Savio C, Zannini L, Micciché F, Masnada D, Nakanishi M, Tauchi H, Komatsu K, Mizutani S, Khanna KK, Chen P, Concannon P, Chessa L, Delia D: Chk2 activation dependence on Nbs1 after DNA damage. Mol Cell Biol 2001;21:5214–5223.
48 Antoccia A, Di Masi A, Maraschio P, Stumm M, Ricordy R, Tanzarella C: G2-phase response in Nijmegen Breakage Syndrome lymphoblastoid cell lines. Cell Prolif 2002;35:93–104.
49 Xu B, Kim S-T, Lim D-S, Kastan BM: Two molecularly distinct G2/M checkpoints are induced by ionizing irradiation. Mol Cell Biol 2002;22:1049–1059.
50 Matsuura K, Balmukhanov T, Tauchi H, Weemaes C, Smeets D, Chrzanowwska K, Endou S, Matsuura S, Komatsu K: Radiation induction of p53 in cells from Nijmegen breakage syndrome is defective but not similar to ataxia telangiectasia. Biochem Biophys Res Commun 1998;24:602–607.
51 Yamazaki V, Wegner RD, Kirchgessner CU: Characterization of cell cycle checkpoint response after ionizing radiation in Nijmegen breakage syndrome cells. Cancer Res 1998;58:2316–2322.
52 Gennery AR, Cant AJ, Jeggo PA: Immunodeficiency associated with DNA repair defects. Clin Exp Immunol 2000;121:1–7.
53 Petrini JH, Donovan JW, Dimare C, Weaver DT: Normal V(D)J coding junction formation in DNA ligase I deficiency syndromes. J Immunol 2001;152:176–183.
54 Harfst E, Cooper S, Neubauer S, Distel L, Grawnder U: Normal V(D)J recombination in cells from patients with Nijmegen breakage syndrome. Mol Immun 2000;37:915–929.
55 Petersen S, Casellas R, Reina-San-Martin B, Chen HT, Difilippantonio MJ, Wilson PC, Hantisch L, Celeste A, Muramatsu M, Pilch DR, Redon C, Ried T, Bonner WM, Honjo T, Nussenzweig MC, Nussenzweig A: AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. Nature 2001;414:660–665.
56 de Jager M, van Noort J, van Gent DC, Dekker C, Kanaar R, Wyman C: Human Rad50/Mre11 is a flexible complex that can tether DNA ends. Mol Cell 2001;8:1129–1135.
57 van Engelen BG, Hiel JA, Gabreels FJ, van der Heuvel DC, van Gent CM, Weemaes CM: Decreased immunoglobulin class switching in Nijmegen breakage syndrome due to the DNA repair defect. Hum Immunol 2001;61:1324–1327.
58 Lahdesmaki A, Taylor AMR, Chrzanowska KH, Pan-Hammarstrom Q: Delineation of the role of the Mre11 complex in class switch recombination. J Biol Chem 2004;279:16479–16487.
59 Zhu J, Petersen S, Tessarollo L, Nussenzweig A: Targeted disruption of the Nijmegen breakage syndrome gene *NBS1* leads to early embryonic lethality in mice. Curr Biol 2001;11:105–109.
60 Reina-San Martin B, Nussenzweig MV, Nussenzweig A, Difilippantonio S: Genomic instability, endoreduplication, and diminished Ig class switch recombination in B cells lacking Nbs1. Proc Natl Acad Sci USA 2005;102:1590–1595.
61 Kracker S, Bergmann Y, Demuth I, Frappart PO, Hildebrand G, Christine R, Wang ZQ, Sperling K, Digweed M, Redbruch A: Nibrin functions in Ig class switch recombination. Proc Natl Acad Sci USA 2005;102:1584–1589.
62 Tauchi H, Matsuura S, Kobayashi J, Sakamoto S, Komatsu K: Nijmegen Breakage Syndrome gene, *NBS1*, and molecular links to factors for genome stability. Oncogene 2002;21:8967–8980.
63 O'Driscoll M, Jeggo PA: Clinical impact of ATR checkpoint signalling failure in humans. Cell Cycle 2003;2:A32–A33.
64 Petrini JH, Stracker TH: The cellular response to DNA double-strand breaks: defining the sensors and mediators. Trends Cell Biol 2003;13:458–462.
65 Nakada D, Matsumoto K, Sugimoto K: ATM-related Tel1 associates with double-strand breaks through an Xrs2 dependent mechanism. Genes Dev 2003;17:1957–1962.
66 Bakkenist CJ, Kastan MB: DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421:499–506.
67 Girard PM, Riballo E, Begg AC, Waugh A, Jeggo PA: Nbs1 promotes ATM dependent phosphorylation events including those required for G1/S arrest. Oncogene 2002;21:4191–4199.
68 Uziel T, Lernthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y: Requirement of the MRN complex for ATM activation by DNA damage: EMBO J 2003;22:5612–5621.

69 Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB: Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. Genes Dev 2004;18:1423–1438.
70 Stiff T, Reis C, Alderton GK, Woodbine L, O'Driscoll M, Jeggo PA: Nbs1 is required for ATR-dependent phosphorylation events. EMBO J 2005;24:199–208.
71 Andreassen PR, D'Andrea AD, Taniguchi T: ATR couples FANCD2 monoubiquitination to the DNA response. Genes Dev 2004;18:1958–1963.
72 Gregory RC, Taniguchi T, D'Andrea AD: Regulation of Fanconi anemia pathway by monoubiquitination. Semin Cancer Biol 2003;13:77–82.
73 Gennery AR, Slatter MA, Bhattacharya A, Barge D, Haigh S, O'Driscoll M, Coleman R, Abinum M, Flood TJ, Cant AJ, Jeggo PA: The clinical and biological overlap between Nijmegen breakage syndrome and Fanconi anemia. Clin Immunol 2004;113:214–219.

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Werner Syndrome, Aging and Cancer

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Abstract

Werner syndrome (WS) is a rare autosomal recessive genetic instability/cancer predisposition disorder that displays many symptoms of premature aging. The mimicry of age-related phenotypes in WS, as well as its dependence on a single defective gene product, has provided the impetus for studying this fascinating disease as a model system for normative aging and its related pathologies such as atherosclerosis, neoplasia, diabetes mellitus, and osteoporosis. The gene product defective in WS, WRN, is a member of the RecQ DNA helicase family that is widely distributed in all kingdoms of life, and is believed to play a central role in genomic stability by preferentially operating on non-canonical DNA structures. Although there have been considerable advances in our understanding of the biochemistry of WRN and its interacting protein partners, the in vivo molecular function(s) of WRN remain(s) elusive. In addition to summarizing the features and clinical progression of WS, the following chapter details our current understanding of the WRN protein with respect to its biochemistry and its interacting protein partners, and considers its putative in vivo roles in various DNA transactions.

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In this article we will review the molecular studies on Werner syndrome, an intriguing disease that may offer clues to human aging and to lineage specificity in human cancers. The major motivation in studying Werner syndrome as a model system comes from its experimental tractability as it involves only one gene product and its functional interactions. In contrast, any attempt in understanding the genetic basis of either ‘normal’ aging processes or cancer will inevitably face a staggering number of interactions in complex genetic networks, making their study an extremely complicated task.

The gene mutated in Werner syndrome is a member of the RecQ DNA helicase family. These enzymes play central roles in maintaining the integrity of the genome while functioning as suppressors of inappropriate recombination.

They are defined by their amino acid similarity to the prototypical *E. coli* RecQ, and are widely distributed across species in all three domains of life. Humans possess five homologs of *E. coli* RecQ, RECQ1, RECQ2/BLM, RECQ3/WRN, RECQ4 and RECQ5. Mutations in three of these, BLM, WRN, and RECQ4, result in genetic instability syndromes, Bloom's syndrome (BS), Werner syndrome (WS), and Rothmund-Thomson syndrome (RTS), respectively, and are manifested by tumor predisposition and/or premature aging. While inherited mutations in BLM helicase are linked with elevations of sister chromatid exchanges and a substantial increase in a wide spectrum of malignancies with the mean age of onset of 24, mutations in the WRN protein are associated with the premature onset of a number of age-related problems and an increased incidence of specific human tumors, especially sarcomas. Thus, WS can be considered a segmental progeroid syndrome (syndromes that mimic some but not all aspects of normative aging) of which early-onset cancer is just one feature [1, 2].

Although WS is a useful model, it must be kept in mind that progeroid syndromes are essentially phenocopies of normal aging. For that reason, it is important to distinguish between observations pertinent to the disease itself and insights that could more generally be applied to processes operating in normal aging and cancer predisposition and progression.

Clinical Features of Werner Syndrome (WS)

Werner syndrome is a rare autosomal recessive genetic instability/cancer predisposition disorder that displays many symptoms of premature aging [3, 4]. It has an estimated frequency of 1 per million in the general population worldwide, though there are nearly 35 WS patients per million in Japan, which harbors 850 of the approximately 1200 cases reported worldwide. However, the prevalence of heterozygous carriers is predicted to be greater than 6 in 1000 [5].

Generally, there is little clinical evidence of abnormality until puberty, when the affected patients fail to demonstrate the adolescent growth spurt. The condition becomes apparent between 15 and 30 years of age, when the other features of premature aging are manifested. These include bilateral ocular cataracts, premature graying and progressive loss of scalp hair, and mild type 2 (late onset) diabetes mellitus. Depletion of subcutaneous adipose tissue gives the appearance of shiny, tight skin on the face and the extremities similar to scleroderma, and ulcers develop over pressure points on the limbs, especially the legs. Osteoporosis, particularly in the long bones of the legs, radiologically characteristic osteosclerosis of the distal bones of the hands and feet, arteriosclerosis, and

hypogonadism are also commonly observed. Further, the deposition of insoluble calcium salts in the soft tissue and blood vessels, as well as the severe calcification of the cardiac valves, is associated with a history of myocardial infarction, and to a lesser degree, cerebral ischemia. Excessive hyaluronic acid excretion that is approximately 10–12 times higher than age-matched controls is observed in all cases. While common epithelial cancers such as lung and colon are no greater than that in controls of similar age, the patients show a high incidence of tumors of mesenchymal origin. Further, multiple distinct primary neoplasms are not uncommon – as many as five malignancies in one patient have been reported. The major causes of death are myocardial infarctions and cancer, and death occurs at a median age of around 47–48 years [6]. Figure 1 illustrates the clinical progression and signs of WS.

While there are significant correlations between the clinical features of WS and normative aging, such as graying of the hair, atherosclerosis and diabetes, it is important to note that there are also a number of important discordances – for instance, the pattern of neoplasms and the distribution of osteoporosis, as well as the infrequency of senile changes in the CNS and the immune system [6, 7]. Regardless of the differences between WS and normative aging, the fact remains that WS mimics most aspects of normal aging and is a classical cancer predisposition syndrome as evidenced by an increased risk of developing cancer.

Cellular Phenotype of WS

There are three consistent cellular defects that have been identified in cultured primary cells isolated from human WS patients: (i) early replicative senescence and cell proliferation defects that have best been defined in skin fibroblasts, (ii) increased sensitivity to DNA damaging agents, and (iii) genetic instability at both the molecular and cytogenetic level [8].

Cultured fibroblasts from WS patients display an extended S phase of the cell cycle and show a decreased average lifespan in culture (approximately 27% of that of normal cells). Further, this replicative decline is not associated with an accelerated loss of telomeres, which are longer than that of senescent normal cells [9, 10]. WS cells exhibit increased sensitivity to DNA damaging agent 4-nitroquinoline 1-oxide (4-NQO) as well as a variety of DNA cross-linking drugs (melphalan, chlorambucil, mitomycin C, cisplatin) and topoisomerase inhibitors (camptothecin, VP-16, amsacrine). In contrast, WS cells are not especially sensitive to UV, γ-irradiation, alkylating agents, bleomycin, and hydroxyurea [8, 11]. Interestingly, heterozygous ES cells show sensitivity to camptothecin that is intermediate between homozygous WS and wild-type cells, suggesting a haploinsufficiency effect [12]. An interesting cytogenetic feature of WS at the chromosomal

Fig. 1. Clinical features of WS. Photographs of two patients displaying the typical features of the Werner syndrome, including bilateral ocular cataracts, premature graying and progressive loss of scalp hair, as well as the appearance of shiny, tight skin, and in panel ***d***, the atropic forearms and elbow ulceration. The photographs show the patients at ages 15 (***a***) and 48 (***b***), and 13 (***c***) and 56 (***d***). The photographs are used with kind permission of Drs. George Martin and Nancy Hanson of the International Registry of Werner Syndrome at the University of Washington.

level is the manifestation of 'variegated translocation mosaicism', defined as pseudodiploidy (46 chromosomes with structural abnormalities) with multiple, variable, clonal chromosome rearrangements, translocations, inversions and extensive deletions in different cell clones [7]. While WS cells do not show an

elevated level of sister chromatid exchanges as is characteristic with Bloom's syndrome cell lines, double strand breaks occur at a higher frequency than in wild type cells [8].

The fact that these features of the WS cell phenotype are experimentally tractable and easily quantifiable underscores the importance of understanding WRN function at the cellular level in providing invaluable insight directly relevant to disease pathogenesis as well as aging.

The *WRN* Gene and Protein

Initial assignment of the *WRN* locus to chromosomal position 8p12 by linkage mapping [13] led to its positional cloning and identification as a member of the RecQ family of DNA helicases [14]. Interestingly, the diverse collection of organismal and cellular phenotypes of WS is caused by the loss-of-function mutations in a single gene product. The WRN protein is a single polypeptide of 1432-amino-acids with a predicted molecular mass of 162 kDa [14]. WRN is a DNA-dependent ATPase that uses the energy from ATP hydrolysis to unwind double-stranded DNA in the 3′ to 5′ direction with respect to the single strand that it binds [15]. However, unlike other known members of the human RecQ family, WRN protein is unique among the five human RecQ members in that it is a bipartite and bifunctional enzyme: in addition to helicase, it also possesses a 3′ to 5′ exonuclease activity with respect to the strand that it degrades [16, 17]. Furthermore, the two functions of the enzyme are functionally and physically separable from each other. Amino acid substitutions that inactivate the exonuclease activity of WRN need not interfere with its helicase function, while mutant proteins with amino acid substitutions or deletions in the helicase/ATPase domain are still able to exonucleolytically digest DNA but fail to unwind it [16, 17]. Figure 2 schematically depicts the domain structure of the WRN protein, as well as the other human RecQ family helicases and the prototypical *E. coli* RecQ.

Interestingly, all of the *WRN* mutations that have been reported in WS patients thus far lead to a truncated gene product lacking the nuclear localization signal [18], consistent with the autosomal recessive nature of the WS. Recent work using single amino acid substitutions that abolish either the helicase or the exonuclease functions of WRN demonstrated that both of these activities must be lost in order to produce recombination and cell survival defects that are characteristic of WS patients [19], a finding further corroborated by the absence of missense mutations in WS patients that selectively abolish either activity of WRN. On the other hand, WRN protein levels in cells from heterozygous carriers are about half of what is seen in healthy individuals [18],

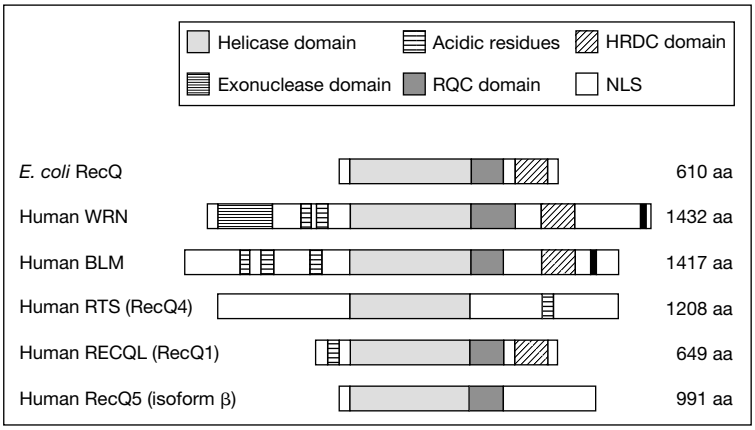

Fig. 2. Comparison of the molecular architecture of the prototypic *E. coli* RecQ helicase and the members of the human RecQ helicase family. Proteins are aligned by their conserved helicase domain. RQC and HRDC domains allude to *Rec**Q** c*onserved and *H*elicase and *R*nase*D C*-terminal domains, respectively, and NLS represents the *n*uclear *l*ocalization *s*ignal-dependent nucleolar targeting sequence. Only the isoform β of the three splice variants of human RecQ5 protein is shown. The sizes of the proteins (in amino acids) are indicated on the right.

conferring an intermediate sensitivity to DNA-damaging agents. At least one common polymorphic variant of the WRN has been shown to have a dramatically reduced catalytic function [20]. Interestingly, several diagnosed WS cases have been shown to express an apparently wild type WRN protein [18], raising the possibility that the existence of mutations in some of the myriad WRN interacting proteins might also cause the WS phenotype.

Biochemistry of WRN Helicase/Exonuclease

Subsequent to its expression and purification from insect cells, WRN protein has been shown to act in vitro as an ATP-dependent DNA helicase with a 3′ to 5′ directionality [15]. While the helicase activity of WRN shows very poor processivity without its interacting protein partners, it has been shown to resolve a variety of DNA substrates (i.e. separate two or more DNA strands by breaking their hydrogen bonds), some of which deviate from the canonical B-form duplex DNA that could potentially interfere with cellular processes such as replication or transcription, thus giving rise to genomic instability. Among the diverse DNA substrates that WRN can unwind are forked DNA molecules,

partial DNA/DNA and DNA/RNA duplexes, partial DNA duplexes with a single-stranded 3′ overhang, and D-loops. Further, WRN can efficiently disrupt quadruplex DNA structures (also called G4 tetraplex DNA) in vitro, which may be widely distributed throughout the genome and are found, among other places, at immunoglobulin switch regions and rDNA gene clusters, as well as at telomeric repeats [21]. It is possible that these structures have specific in vivo functions in the regulation of gene expression or genetic stability. In addition, WRN can resolve triplex DNAs (which have been demonstrated both in chromosomes and nuclei [22]), and is capable of branch-migrating Holliday junctions over several kilobases, a remarkable feat considering its poor processivity [2, 8, 11]. Taken together, these substrate requirements suggest that a major function of WRN is to alleviate blocks during DNA synthetic processes.

Similar to its helicase activity, WRN exonuclease displays a 3′ to 5′ directionality and low processivity [16, 17]. Although WRN exonuclease needs the presence of 3′ recessed termini for activity, this requirement is relaxed if the substrate can adopt certain defined structures such as a bubble-containing duplex DNA, DNA with single-stranded loop, stem-loop DNA molecules, as well as three-way and four-way DNA junctions; in these cases, WRN initiates digestion from blunt ends [2, 8, 11]. Since both the helicase and the exonuclease activities of the WRN protein reside on the same polypeptide [17], this preference for alternative DNA structures is not surprising, and may in fact reflect an enhanced binding that facilitates activity. Based on the observation that a partial duplex with a 3′ mismatched terminus is a better substrate for WRN exonuclease than an otherwise identical non-mismatched molecule, it has been postulated that WRN may play a role in proofreading akin to some DNA polymerases. Figure 3 schematically depicts some of the preferred substrates of WRN.

Despite the wealth of biochemical data, it is not known whether the helicase and exonuclease activities of the WRN protein function coordinately in a common molecular pathway. While similar binding affinities and substrate preferences suggest coordinate action, it is also possible that the separate activities of the protein may be involved in discrete steps of a single pathway, sequentially playing independent roles. An alternative, albeit less likely scenario, is that the helicase and exonuclease activities of WRN operate separately in two distinct DNA metabolic pathways.

WRN Protein Partners

The diverse cellular and organismal phenotypes of WS suggest that WRN may play a role in multiple cellular processes involving physical and functional associations with other proteins. Indeed, consistent with the bipartite and

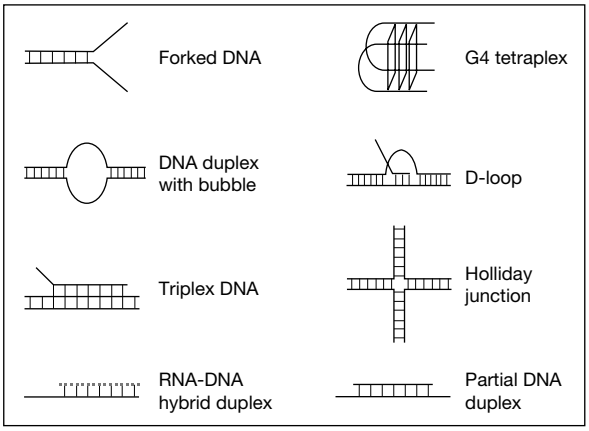

Fig. 3. Various substrates of WRN. WRN shows substrate specificity towards alternate DNA structures thought to exist in vivo as intermediates in specific DNA transactions such as replication (forked DNA), recombination (Holliday junction, triplex and tetraplex DNA), and repair (partial duplex DNA with single-stranded bubble).

bifunctional nature of WRN and its many substrates that resemble numerous intermediates in DNA replication, recombination, and repair processes, the search for the cellular functions of WRN has uncovered a large number of protein partners that participate in various aspects of DNA metabolism. Below, we review the physical and functional interactions of WRN with protein partners as evidence of WRN participation in different aspects of DNA metabolism. Unfortunately, the existence of a large number of WRN-interacting proteins that participate in multiple DNA synthetic pathways have so far prevented a clear definition of the cellular function of WRN based on associations.

Early work demonstrated that WRN copurified with a 17 S multiprotein replication complex that contained several replication factors, including PCNA and topoisomerase I that formed a physical complex with WRN [23]. Subsequent reports from various laboratories identified a number of additional WRN-associating replication proteins, including RPA, flap endonuclease I (FEN-I), and most interestingly DNA polymerase-δ [2, 8, 11]. The interaction of WRN with DNA polymerase-δ allows the polymerase to replicate through alternative DNA structures that normally impede the progression of the replication fork [24]. This observation is consistent with the substrate specificity of the WRN helicase/exonuclease, and suggests that WRN might be involved in removing replication blocks during DNA synthesis. However, all these WRN protein partners are also involved in DNA repair, especially the long patch base excision repair (BER), raising the possibility that WRN may play a dual role

in both DNA replication and repair. Indeed, it has been shown that WRN physically and functionally interacts with other BER proteins such as DNA polymerase-β and poly(ADP-ribose)polymerase 1 (PARP-1), as well as Ku and DNA-dependent protein kinase (DNA-PK), which are required for double strand break repair via the non-homologous end joining (NHEJ) pathway [2, 8, 11, 25].

Recent studies that were initiated on the premises that tumor suppressors may regulate both tumorigenesis and cellular aging and that WRN and p53 may possibly be linked in a common pathway determining cell aging revealed that p53 directly associates with WRN in vitro and in vivo. This association modulates the activity of both partners: while the helicase and the exonuclease activities of WRN are inhibited by its interaction with p53, in a reciprocal manner WRN augments p53-mediated transcription from p53-responsive elements [8, 11]. The p53-WRN interaction may be crucial in p53-mediated apoptosis in S phase cells, as evidenced by the attenuation of this pathway in WS cells [26], which could be associated with the cancer predisposition phenotype of WS patients.

Another clue to the potential in vivo role of WRN comes from its interaction with proteins involved in telomere maintenance. Early statistical evidence indicating an accelerated shortening of telomeres in serially passaged WS cultures, together with the indication that the loss of telomeric DNA may determine the onset of replicative senescence, provided the initial impetus for the investigation of the role of WRN in telomere maintenance [10]. The fact that the preferred substrates of WRN such as D-loops and G-quadruplex DNA are formed at telomeric ends, and that WRN binds and functionally interacts with the critical telomere binding and maintenance protein TRF-2 while colocalizing with both TRF-1 and TRF-2 in nuclear foci of immortalized human cells lacking telomerase support the hypothesis that WRN may play a role in processing of telomeric end structures [2, 11].

In vivo Role(s) of WRN

The motivation for the study of WRN protein is based on the premise that WS, as a useful model system involving a single gene product, can promote the formulation of directed and experimentally tractable mechanistic insights into the process of normal aging as well as age-associated diseases. However, with our ever expanding knowledge of its intricate biochemistry and cell biology, its multiple interacting protein partners and the complex phenotypic manifestations its absence creates, it is becoming clear that WRN participates in more than a single DNA metabolic pathway. Yet, most lines of evidence presented so far are compatible with an overarching role for WRN in the resolution of

alternative DNA structures in a variety of DNA synthetic processes. For example, it is hypothesized that WRN clears the path for the replicative apparatus during DNA replication by resolving alternative DNA structures that would otherwise impede the progression of the replication fork. WRN protein, with its dual helicase/exonuclease functionality, has been shown to preferentially bind and efficiently process non-canonical DNA structures. Giving further credence to this is the fact that WRN interacts with a variety of replication proteins and that WS cells display a prolonged S-phase. However, since many of these proteins are also involved in DNA repair, an exclusively replication-specific role to WRN cannot be assigned. In addition to the proteins with dual roles in DNA replication and repair, WRN interacts with bona fide repair proteins, and WS cells have been shown to accumulate chromosomal rearrangements and somatic mutations at an increased rate in an age-dependent manner. Further, WS lymphoblastoid cell lines display reduced levels of gene-specific and strand specific repair of UV damage, and are impaired in their ability to resolve mitotic recombination products. Then again, in addition to repair, these structural intermediates arise in a variety of DNA metabolic processes such as replication, repair, and recombination. Finally, the increased loss of telomeres in WS cells and the correlation between aging and telomeric attrition suggest a role for WRN in telomere maintenance. Further supporting this link are the association of WRN with telomere repeat binding factors TRF1 and TRF2, as well as the high specificity of WRN for G-rich alternative DNA structures found in telomeres. Additional evidence for the involvement of WRN in telomere maintenance comes from mouse studies where late generation telomerase deficient mice with both short, dysfunctional telomeres and a mutant WRN allele have exhibited many of the clinical features observed in WS patients. Created independently by two laboratories to investigate the role of telomere attrition in the pathogenesis of WS, these telomerase RNA template deficient (or *mTerc*$^{-/-}$) mice in a WRN null background showed telomere length-dependent manifestation of classical WS symptoms such as premature death, hair graying, alopecia, osteoporosis with skeletal fractures, cataracts and type II diabetes accompanied by enhanced telomere dysfunction, including increased chromosomal fusions and non-reciprocal translocations [27, 28]. Taken together, these data indicate a direct link between telomere shortening and the manifestation of the WS and suggest a role for WRN in telomere maintenance.

Conclusion

Werner syndrome hides important clues to the biology of aging and age-associated diseases. While our detailed analyses of the biochemistry of the

encoded protein have defined its function as a helicase and exonuclease, it has failed to establish its precise role in in vivo DNA transactions. The large number of physical and functional interactors of WRN suggests that WRN could be a sticky protein that is involved in multiple DNA synthetic processes. Again, these associations have so far failed to yield definitive mechanistic insights into cellular pathways. It seems to be the case, however, that regardless of the precise mechanistic function of WRN in vivo, its loss leads the cell down a path of genomic instability, possibly resulting from the persistence of recombination substrates that are generated during stalled replication, or as intermediates of telomere maintenance. While their successful resolution suppresses genomic instability and ensures cell survival, these potentially toxic intermediates elicit both DNA damage and apoptotic response in the absence of WRN.

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References

1 Hickson ID: RecQ helicases: caretakers of the genome. Nat Rev Cancer 2003;3:169–178.
2 Opresko PL, Cheng WH, Bohr VA: Junction of RecQ helicase biochemistry and human disease. J Biol Chem 2004;279:18099–18102.
3 Epstein CJ, Martin GM, Schultz AL, Motulsky AG: Werner's syndrome a review of its symptomatology, natural history, pathologic features, genetics and relationship to the natural aging process. Medicine (Baltimore) 1966;45:177–221.
4 Martin GM: Genetic syndromes in man with potential relevance to the pathobiology of aging. Birth Defects Orig Artic Ser 1978;14:5–39.
5 Miki T, Nakura J, Ye L, Mitsuda N, Morishima A, Sato N, Kamino K, Ogihara T: Molecular and epidemiological studies of Werner syndrome in the Japanese population. Mech Ageing Dev 1997;98:255–265.
6 Martin GM: Genetic modulation of senescent phenotypes in *Homo sapiens*. Cell 2005;120: 523–532.
7 Ostler EL, Wallis CV, Sheerin AN, Faragher RG: A model for the phenotypic presentation of Werner's syndrome. Exp Gerontol 2002;37:285–292.
8 Bachrati CZ, Hickson ID: RecQ helicases: suppressors of tumorigenesis and premature aging. Biochem J 2003;374(Pt 3):577–606.
9 Salk D, Bryant E, Au K, Hoehn H, Martin GM: Systematic growth studies, cocultivation, and cell hybridization studies of Werner syndrome cultured skin fibroblasts. Hum Genet 1981;58: 310–316.

10 Schulz VP, Zakian VA, Ogburn CE, McKay J, Jarzebowicz AA, Edland SD, Martin GM: Accelerated loss of telomeric repeats may not explain accelerated replicative decline of Werner syndrome cells. Hum Genet 1996;97:750–754.
11 Fry M: The Werner syndrome helicase-nuclease – one protein, many mysteries. Sci Aging Knowledge Environ 2002(13):re2.
12 Lebel M, Leder P: A deletion within the murine Werner syndrome helicase induces sensitivity to inhibitors of topoisomerase and loss of cellular proliferative capacity. Proc Natl Acad Sci USA 1998;95:13097–13102.
13 Goto M, Rubenstein M, Weber J, Woods K, Drayna D: Genetic linkage of Werner's syndrome to five markers on chromosome 8. Nature 1992;355:735–738.
14 Yu CE, Oshima J, Fu YH, Wijsman EM, Hisama F, Alisch R, Matthews S, Nakura J, Miki T, Ouais S, et al: Positional cloning of the Werner's syndrome gene. Science 1996;272:258–262.
15 Gray MD, Shen JC, Kamath-Loeb AS, Blank A, Sopher BL, Martin GM, Oshima J, Loeb LA: The Werner syndrome protein is a DNA helicase. Nat Genet 1997;17:100–103.
16 Huang S, Li B, Gray MD, Oshima J, Mian IS, Campisi J: The premature ageing syndrome protein, WRN, is a 3′→5′ exonuclease. Nat Genet 1998;20:114–116.
17 Shen JC, Gray MD, Oshima J, Kamath-Loeb AS, Fry M, Loeb LA: Werner syndrome protein. I. DNA helicase and DNA exonuclease reside on the same polypeptide. J Biol Chem 1998;273: 34139–34144.
18 Goto M, Yamabe Y, Shiratori M, Okada M, Kawabe T, Matsumoto T, Sugimoto M, Furuichi Y: Immunological diagnosis of Werner syndrome by down-regulated and truncated gene products. Hum Genet 1999;105:301–307.
19 Swanson C, Saintigny Y, Emond MJ, Monnat RJ Jr: The Werner syndrome protein has separable recombination and survival functions. DNA Repair (Amst) 2004;3:475–482.
20 Kamath-Loeb AS, Welcsh P, Waite M, Adman ET, Loeb LA: The enzymatic activities of the Werner syndrome protein are disabled by the amino acid polymorphism R834C. J Biol Chem 2004;279:55499–55505.
21 Simonsson T: G-quadruplex DNA structures – variations on a theme. Biol Chem 2001;382: 621–628.
22 Lee JS, Burkholder GD, Latimer LJ, Haug BL, Braun RP: A monoclonal antibody to triplex DNA binds to eucaryotic chromosomes. Nucleic Acids Res 1987;15:1047–1061.
23 Lebel M, Spillare EA, Harris CC, Leder P: The Werner syndrome gene product co-purifies with the DNA replication complex and interacts with PCNA and topoisomerase I. J Biol Chem 1999;274:37795–37799.
24 Kamath-Loeb AS, Loeb LA, Johansson E, Burgers PM, Fry M: Interactions between the Werner syndrome helicase and DNA polymerase delta specifically facilitate copying of tetraplex and hairpin structures of the $d(CGG)_n$ trinucleotide repeat sequence. J Biol Chem 2001;276:16439–16446.
25 Lee JW, Harrigan J, Opresko PL, Bohr VA: Pathways and functions of the Werner syndrome protein. Mech Ageing Dev 2005;126:79–86.
26 Spillare EA, Robles AI, Wang XW, Shen JC, Yu CE, Schellenberg GD, Harris CC: p53-mediated apoptosis is attenuated in Werner syndrome cells. Genes Dev 1999;13:1355–1360.
27 Du X, Shen J, Kugan N, Furth EE, Lombard DB, Cheung C, Pak S, Luo G, Pignolo RJ, DePinho RA, et al: Telomere shortening exposes functions for the mouse Werner and Bloom syndrome genes. Mol Cell Biol 2004;24:8437–8446.
28 Chang S, Multani AS, Cabrera NG, Naylor ML, Laud P, Lombard D, Pathak S, Guarente L, DePinho RA: Essential role of limiting telomeres in the pathogenesis of Werner syndrome. Nat Genet 2004;36:877–882.

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Fanconi Anemia: Causes and Consequences of Genetic Instability

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Abstract

Fanconi anemia (FA) is a rare recessive disease that reflects the cellular and phenotypic consequences of genetic instability: growth retardation, congenital malformations, bone marrow failure, high risk of neoplasia, and premature aging. At the cellular level, manifestations of genetic instability include chromosomal breakage, cell cycle disturbance, and increased somatic mutation rates. FA cells are exquisitely sensitive towards oxygen and alkylating drugs such as mitomycin C or diepoxybutane, pointing to a function of FA genes in the defense against reactive oxygen species and other DNA damaging agents. FA is caused by biallelic mutations in at least 12 different genes which appear to function in the maintenance of genomic stability. Eight of the FA proteins form a nuclear core complex with a catalytic function involving ubiquitination of the central FANCD2 protein. The posttranslational modification of FANCD2 promotes its accumulation in nuclear foci, together with known DNA maintenance proteins such as BRCA1, BRCA2, and the RAD51 recombinase. Biallelic mutations in *BRCA2* cause a severe FA-like phenotype, as do biallelic mutations in *FANCD2*. In fact, only leaky or hypomorphic mutations in this central group of FA genes appear to be compatible with life birth and survival. The newly discovered *FANCJ* (= *BRIP1*) and *FANCM* (= *Hef*) genes correspond to known DNA-maintenance genes (helicase resp. helicase-associated endonuclease for fork-structured DNA). These genes provide the most convincing evidence to date of a direct involvement of FA genes in DNA repair functions associated with the resolution of DNA crosslinks and stalled replication forks. Even though genetic instability caused by mutational inactivation of the *FANC* genes has detrimental effects for the majority of FA patients, around 20% of patients appear to benefit from genetic instability since genetic instability also increases the chance of somatic reversion of their constitutional mutations. Intragenic crossover, gene conversion, back mutation and compensating mutations in *cis* have all been observed in revertant, and, consequently, mosaic FA-patients, leading to improved bone marrow function. There probably is no other experiment of nature in our species in which causes and consequences of genetic instability, including the role of reactive oxygen species, can be better documented and explored than in FA.

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In recent years, the maintenance of genome integrity has emerged as a major determinant of organismic longevity and cellular viability [1]. Genetic instability increases the risk of neoplastic transformation and contributes to premature aging [2–4]. The importance of a stable genome for healthy aging is exemplified by numerous genetic instability syndromes which are caused by defective caretaker genes [5–7]. Classical examples are the chromosome breakage syndromes such as Bloom syndrome, Werner syndrome, Ataxia telangiectasia, and Fanconi anemia. Instability of telomeres due to defective telomerase or telomerase-associated dyskerin causes Dyskeratosis congenita, a premature aging syndrome with pigmentation changes, leukoplakia and aplastic anemia [8]. The least specific type of nuclear or chromatin instability is caused by mutations in the lamin A/C gene which encodes the inner lamina of the nuclear membrane. Destabilization of the nuclear membrane by defective lamin A/C leads to global disturbance of gene expression affecting many cell types. The resulting clinical phenotypes are referred to as 'laminopathies' and include various myopathies, lipodystrophies, neuropathies and, most prominently, the Hutchinson-Gilford juvenile progeria syndrome in which many organ systems and cell types exhibit the detrimental effects of nuclear instability [9]. The present chapter deals with one of the most enigmatic human chromosome breakage syndromes, Fanconi anemia (FA), a rare recessive disease whose molecular basis has been elucidated in recent years [10–13]. FA serves as a paradigm for the far reaching and mostly detrimental effects of genetic instability, including congenital malformations, neoplasia and premature aging. FA genes appear to be centrally important in maintaining a stable genome. At the same time, FA serves as an example of rare beneficial effects of genetic instability, i.e. increased likelihood of somatic reversion. Such self-corrections of disease causing mutations ('natural gene therapy') have been described in only a handful of human diseases [14]. FA is a prime example of the different types of molecular events that lead to the restoration of heterozygosity, and thereby normal function, of the self-corrected cell types in individuals constitutionally homozygous or compound heterozygous for disease causing gene mutations.

The Fanconi Anemia Phenotype

Fanconi anemia is a chromosome instability disorder characterized by diverse clinical features including developmental abnormalities, progressive bone marrow failure and increased risk of neoplasia [15] (see also table 1). The first case report was published in 1927 by the Swiss paediatrician Guido Fanconi. He described a hereditary form of progressive aplastic anemia in three children with specific somatic abnormalities. Since then, more than 1300 FA

Table 1. Phenotypic features of Fanconi anemia

Clinical phenotype	Cellular phenotype
1) Developmental abnormalities • pre- and postnatal growth retardation • microcephaly • microphthalmia • skeletal malformations (radial ray defects) • malformation of inner organs (kidney and heart) and auditory system • hypogonadism and reduced fertility • hyperpigmentation, hypopigmentation, café au lait spots 2) Progressive bone marrow failure 3) Cancer predisposition (AML, squamous cell carcinomas)	1) Spontaneous chromosomal instability 2) Spontaneous arrest in the G2 phase of the cell cycle 3) Hypersensitivity against DNA crosslinking agents such as mitomycin C, diepoxybutane, cisplatin, psoralen and nitrogen mustard 4) Hypersensitivity against oxygen

patients belonging to all ethnic groups have been diagnosed [16]. It is estimated that 1 in 200,000 people are affected (i.e. homozygotes or compound heterozygotes), and the carrier (heterozygote) frequency is estimated at 1 in 300 [17]. Higher frequencies of patients reflecting carrier frequencies $>1\%$ are encountered in certain populations due to founder and inbreeding effects [17–19].

Average life expectancy of FA patients is around 20 years, and patients reaching the age of 50 are exceedingly rare [20]. 70% of the patients show developmental defects including short stature, radial ray defects, pigmentary changes and urogenital malformations [21]. Endocrine abnormalities such as hyperinsulinemia, growth hormone insufficiency, and hypothyroidism occur in more than 80% of classical FA patients [22]. Bone marrow failure and high risk of myelodysplastic syndrome, myelogenous leukemia and squamous cell carcinomas account for the reduced life expectancy [23]. The classical clinical course is marked by childhood onset of bone marrow failure which first manifests as low platelet counts, complicated by transfusion-dependent anemia and irreversible pancytopenia in the first two decades of life. However, clinical course and clinical phenotype are highly variable both within and between families. Revertant mosaicism may account for some of this variability [24, 25], and 'mild' mutations may account for a number of patients that are diagnosed incidentally or as adults when they present with MDS, AML or squamous cell carcinomas [26]. Treatment is mostly supportive and includes blood product substitution and stimulation of bone marrow cell growth by androgens, hydrocortisone and cytokines; the only curative treatment is bone marrow transplantation,

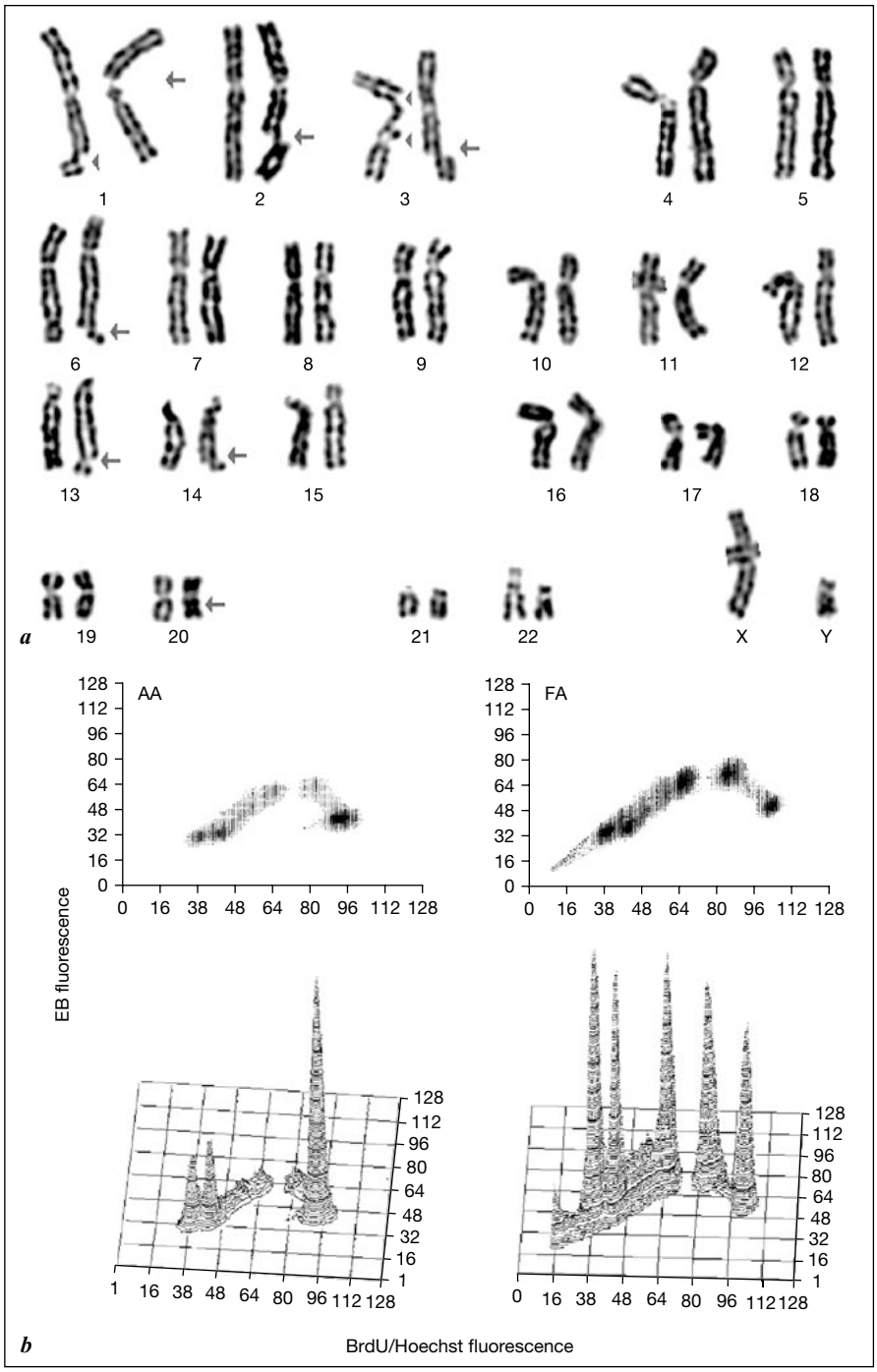
Table 2. Laboratory tests for the confirmation of Fanconi anemia

Test system	Procedure	Advantage/Disadvantage
Chromosome breakage test	72 h blood lymphocyte culture in the presence of DEB, MMC, or NM	high sensitivity; reliable detection of mosaicism, but experience required and labor intensive
Cell cycle test	single or dual parameter flowcytometry; measures G_2-phase accumulations	fast and accurate screening test, but detection of mosaicism is difficult
FANCD2 (posttranslational modification)	immunoblot using antibodies detects FANCD2-S (native) and monoubiquitinated isoform (FANCD2-L)	identifies patients with defects in FANCD2 and in FA genes necessary for FANCD2 monoubiquitination (FA core complex genes); requires cycling cells; difficult with fibroblasts
DNA-test (genomic DNA)	prescreening with DHPLC, exon scanning sequencing	works well with FA genes except FANCD2 (pseudogenes) and FANCA (frequent large deletions; detection requires quantitative techniques)
DNA-test (cDNA)	cDNA-sequencing of RT-PCR products	detects multiple types of mutations; requires high quality mRNA, detects expressed genes only; misses large deletions
Glycophorin-test	analysis of somatic mutation frequency using RBC	provides evidence for ongoing mutation; requires heterozygosity for the MN blood group

but a high proportion of successfully transplanted patients develop solid cancers later in life [27].

The FA Cellular Phenotype

The FA cellular phenotype reflects the underlying genetic instability which can be assessed by a variety of assays. The most frequently applied tests are listed in table 2. Under ambient-air cell culture conditions FA cells show elevated spontaneous chromosomal breakage rates with a predominance of chromatid-type lesions [28] which probably reflect the innate hypersensitivity of FA cells towards oxygen [29]. Glycophorin assays provide direct proof of ongoing and increased somatic mutation [30]. Another hallmark feature of FA cells is their hypersensitivity towards bifunctional alkylating and crosslinking agents such as mitomycin C, diepoxybutane or nitrogen mustard [31, 32]. In response to these agents FA cells show elevated rates of chromatid breaks and chromatid exchanges (cf. fig. 1a). As a consequence of damaged and/or misrepaired DNA, FA cells accumulate with 4N DNA content at the S/G_2 phase border of the cell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cycle [33–36]. The characteristic cell cycle disturbance can be used as a diagnostic test ([37]; fig. 1b).

Unlike any other genetic instability syndrome, FA cells are hypersensitive towards oxygen [29] and grow poorly in conventional cell culture incubators with ambient oxygen tension. However, in vitro growth and cloning of FA cells can be restored to near normal under hypoxic cell culture conditions [38]. In contrast to ATM, FA cells are not markedly hypersensitive towards ionizing radiation under (physiological) low-oxygen conditions, nor do they show consistent hypersensitivity towards UV irradiation [39]. This may be different when FA cells are exposed to ambient oxygen tension and assessed via the single cell electrophoresis (comet) assay [40]. The unique combination of cellular resistance and cellular sensitivities sets FA apart from the other genetic instability syndromes. Hypersensitivity to crosslinking agents and oxygen points towards an important role of FA genes in the defence of nuclear stability and integrity. Together with genes such as *XP*, *ATM*, *NBS1*, *RAD50*, *MRE11*, *BRCA1*, *BRCA2* and *p53*, the FA family of genes has been appropriately included in the category of 'caretaker' or 'guardian of the genome' genes [10].

Genetic Heterogeneity as a Hallmark of Fanconi Anemia

So far, 12 complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, L and M) have been defined via somatic cell fusion [41] and 11 of the corresponding genes (denoted as FANC-A, B, C, D1, D2, E, F, G, J, L, M) have been identified (table 3; [42–46]). Expression cloning led to the identification of FANC-A, C, E, F, and G, whereas a positional cloning strategy was used for the isolation of FANC-A, D2, and J. Three FA-genes (FANC-B, L and M) have been defined via biochemical isolation of proteins, and the *FANCD1* gene was found via a classical candidate gene approach. Interestingly, both the *FANCJ* and *FANCM* genes were suggested as genuine FA genes by the MMC sensitivity of their mutant orthologs

Fig. 1. a Chromatid-type chromosome lesions (arrows/arrowheads) as cytogenetic hallmarks of spontaneous and induced chromosome fragility in Fanconi anemia (FA). Blood lymphocyte karyotype stained with Giemsa. ***b*** Cell cycle alterations as flowcytometric hallmarks of FA. The upper panels show bivariate flowcytograms of 72-h peripheral blood mononuclear blood cell cultures grown in the presence of a lectin mitogen (PHA) and the base analog bromodeoxyuridine (BrdU). Double staining with ethidium bromide (EB) (Y-axis) and the Hoechst 33258 dye (X-axis) resolves the distribution of cells throughout the G_1, S and G_2/M phases of three consecutive cell cycles. In contrast to non-genetic aplastic anemia (AA), cells from patients with FA accumulate in the G_2-phases of consecutive cell cycles. The three-dimensional representation of the bivariate cytogram data (bottom panels) emphasizes the cell cycle differences between the non-genetic and the genetic form of aplastic anemia. For details of the cell cycle testing see [37].

Table 3. Fanconi anemia (FA) complementation groups and genes

Complementation group	Frequency in FA[a]	Gene nomenclature	Exons	Protein size [kDa]	mono-Ub of FANCD2
FA-A	66	*FANCA*	43	163	no
FA-B	1	*FANCB*	10	95	no
FA-C	10	*FANCC*	15	63	no
FA-D1	3	*FANCD1/BRCA2*	28	384	yes
FA-D2	3	*FANCD2*	44	155 (162)	–
FA-E	2	*FANCE*	10	58	no
FA-F	2	*FANCF*	1	42	no
FA-G	9	*FANCG/XRCC9*	14	68	no
FA-I	2	(*FANCI*)	?	?	no
FA-J	2	*FANCJ/BRIP1*	20	140	yes
FA-L	<1	*FANCL*	14	43	no
FA-M	<1	*FANCM/Hef*	23	230	no

[a]Relative prevalence of FA complementation groups according to Levitus et al. [41].

in the chicken DT40 cell line [46, 47]. *FANCB* is the only X-linked FA gene, all others are located on autosomal chromosomes (fig. 2). Five FA genes were found to correspond to previously identified genes: *FANCG* to *XRCC9*, *FANCD1* to *BRCA2*, *FANCL* to *PHF9*, *FANCJ* to *BRIP1/BACH1*, and *FANCM* to *Hef*. This identity strongly supports the caretaker role of the FA genes, since XRCC9, BRCA2, BRIP1 and Hef are known to be involved in DNA-maintenance pathways. Biallelic mutations in *BRCA2/FANCD1* cause an unusually severe type of disease with early childhood leukemia and solid tumors preceding onset of bone marrow failure. Most of these patients will never be diagnosed as FA since they succumb to their malignancies prior to onset of pancytopenia. Some authors therefore consider patients with biallelic mutations in *FANCD1/BRCA2* 'FA-like' rather than genuine FA [48, 49]. *Brca2* knock-out mice are embryonic lethals, and it appears that only hypomorphic mutations in *FANCD1/BRCA2* are compatible with survival in humans [50, 51].

Complementation studies using somatic cell fusion or retroviral transduction have assigned around 60% of FA patients to complementation group A, 8–10% each to groups C and G, and 3% to group D2. The other complementation groups account for less than 2% of the patients (cf. table 3; [41, 52, 53]). With the exception of *FANCD1/BRCA2*, *FANCD2*, *FANCL*, *FANCJ/BRIP1* and *FANCM/Hef*, the other FA genes have arisen rather recently in vertebrate evolution [10]. *FANCL* was shown to evolutionarily co-exist with *FANCD2* in several

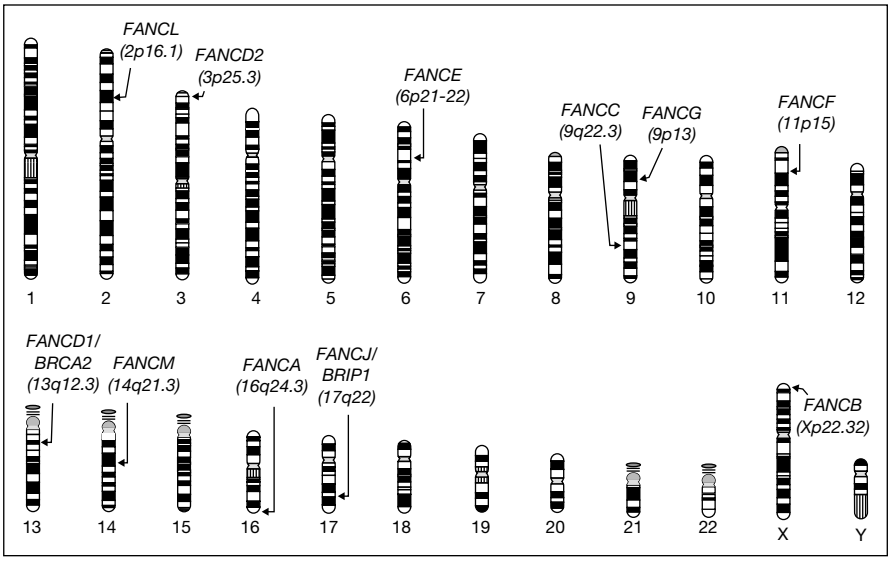

Fig. 2. Human karyotype map with locations of the 11 Fanconi anemia (FA) genes known to date. FA genes are denoted with the prefix 'FANC' and alphabetical letters. Note that there are two types of *FANCD* genes: *FANCD1* (corresponding to *BRCA2*) and *FANCD2*. Chromosome 9 is the only chromosome harboring two FA genes (*FANCG* and *FANCC*), and *FANCB* is the only X-chromosomal FA gene.

species, and it is the only FA gene with proven enzymatic (ubiquitin E3 ligase) activity [54]. The FANCL protein is part of the FA core complex and contains three WD40 repeats known as sites of protein–protein interaction. At the C-terminus FANCL has a PHD zinc finger motif with presumptive E3 ligase activity. Meetei et al. demonstrated that FANCL is able to ubiquitinate itself in vitro and that lack of FANCL results in defective FANCD2 ubiquitination [55]. The crucial role of FANCL in the FA/BRCA-pathway is highlighted by the fact that there are only two FA-L patients known to date, both with hypomorphic mutations (Kalb et al., manuscript in preparation). Because of the lack of evolutionary homologies among the other FA genes, and because of a notable absence of signature motifs at the DNA and protein levels, it remains unclear whether, in addition to their role in the FA core complex (see below), the FA genes serve other specific functions. However, some of the FA genes are highly expressed in spermatogenesis and oogenesis [56], and the location of *FANCC* and *FANCG* on human chromosome 9 within regions that are orthologous to the avian Z chromosome implies a meiotic function (Nanda et al., unpublished observations, 2005). An important role of FA genes in gametogenesis is further suggested by

impaired fertility of male FA patients, failure of spermatogenesis in FA knockout mice [57] and physical association of some of the FA proteins with the synaptonemal complex [58]. A plethora of putative special functions have been ascribed to the FANCC proteins, including interaction with NADPH cytochrome P450 reductase, maintenance of a DNA damage-induced G_2 checkpoint [59], and regulation of genes involved in differentiation and inflammation [60]. Most importantly, it is now firmly established that the proteins encoded by at least 8 of the FA genes participate in the formation of a nuclear protein complex (*FA core complex*) which is assembled in response to DNA damage [61, 62].

Molecular Function of the FA Family of Genes

Since the discovery of the first FA gene (*FANCC*) in 1992 it took nearly 10 years until the identification of the *FANCD2* gene provided a functional link between the FA- and the BRCA caretaker pathways [58, 63]. In response to DNA damage, eight of the known (FANC-A, B, C, E, F, G, L, and M), and a single as yet unidentified FA protein (FAAP 100) assemble into a nuclear complex (*FA core complex*) whose central function appears to be the monoubiquitination of FANCD2 at amino acid residue K561 [55, 64, 65]. Complex formation begins with FANCA and FANCG multimerization, particularly in response to oxidative stress [66]. In addition, both FANCG and FANCF appear to play a key role in the assembly process. The FANCL-protein has ubiquitin ligase activity and is thought to act as catalytic subunit of the FA core complex. Monoubiquination of FANCD2 can be used as a functional test of the integrity of the FA core complex proteins [67]. As shown in figure 3, a defect in one of the core complex genes causes failure of monoubiquination of FANCD2 such that immunoblotting detects only the smaller of the FANCD2 isoforms (S band). Monoubiquitination of FANCD2 reflects functionally intact core complex genes and yields the larger isoform (FANCD2-L). The larger isoform FANCD2-L (corresponding to an activated FANCD2 protein) is targeted to nuclear foci containing BRCA1, BRCA2/FANCD1 and RAD51 known to be involved in recombinational DNA repair [13, 68–70]. Figure 4 illustrates the current view of how FA proteins react in response to DNA damage, emphasizing the role of the FA-D2 protein.

FANCD2 as a Central Effector of the FA/BRCA Network

Several lines of evidence support a key role of FANCD2 in the FA/BRCA caretaker network. First, FANCD2 interacts with members of the FA core complex and with BRCA2, a known player of homology directed DNA repair. Second, as already mentioned, next to FANCL, J and M, FANCD2 is a highly conserved FA gene with orthologs in organisms like *Arabidopsis*, *Caenorhabditis elegans*, drosophila and the zebrafish [63, 71, 72]. *Fancd2*-deficient zebrafish embryos exhibit features reminiscent of the human FA phenotype, including shortened body

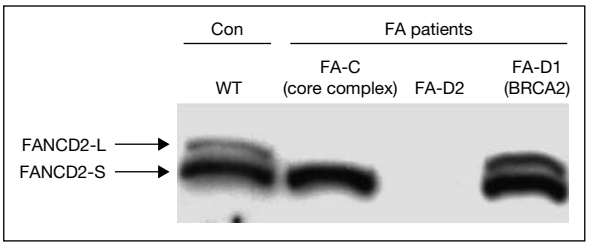

Fig. 3. Westernblots showing examples of unmodified (FANCD2-S) and ubiquitinated (FANCD2-L) isoforms of the human FA-D2 protein. From left to right: following mitomycin C induction, healthy donors (WT) show both the S and the L band, indicating proficient function of the FA core complex proteins. In contrast, defective core complex proteins (e.g. FA-C) are unable to ubiquitinate FANCD2 (second lane). Immunoblotting detects little or no FANCD2 protein in patients carrying biallelic mutations in the *FANCD2* gene (third lane), whereas patients with defects in FA-genes downstream of *FANCD2* (such as *FANCD1/BRCA2*) display both the L and the S band owing to normal function of the core complex proteins (lane four).

length, microcephaly and microphthalmia. These observations emphasize the essential role of an intact *FANCD2* gene during embryogenesis [72]. Third, the activated form of FANCD2 (FANCD2-L) is targeted to sites of DNA-damage and colocalizes with BRCA1, BRCA2, RAD51, PCNA, ATR and probably others. FANCD2-L directly associates with chromatin at the site of DNA repair and is deubiquitinated by USP1 [73]. Depletion of the *Fancd2* ortholog in *C. elegans* causes sensitivity to crosslinking agents and drop in progeny survival, implying an essential role in the DNA damage response [74]. Fourth, there are no FA-D2 patients known to date who carry biallelic null mutations which indicates that only hypomorphic mutations in FANCD2 are compatible with survival [75]. This observation renders the importance of FANCD2 equivalent to that of FANCD1/BRCA2 for which biallelic null mutations appear to be lethal in humans [50]. Like FANCD2, FANCD1/BRCA2 is involved in the stabilization of replication forks [76], and both FANCD1/BRCA2 and FANCD2 are DNA-binding proteins with a structure specific affinity to branch points and free DNA ends [77].

In addition to FANCD1 and FANCD2, the recently discovered DEAH-box containing DNA helicase (= FANCJ) and the helicase-associated endonuclease for fork-structured DNA Hef (= FANCM) appear to interact directly with DNA [45]. In contrast to most of the other FA core complex genes, there is compelling evidence for the direct involvement of these newly discovered FA genes in DNA-repair functions: like the genes underlying Werner, Bloom, Rothmund-Thomson and Rapadilino genetic instability syndromes, FANCJ/BRIP1 is a member of the RecQ DEAH helicase family of genes that unwind DNA in $5' \rightarrow 3'$ direction. The highly conserved RecQ-type helicases are capable of unwinding Holliday

junctions formed during homologous replication or during the resolution of stalled replication forks [44]. Likewise, FANCM/Hef has sequence similiarities to known human DNA repair proteins such as ERCC4 and XPF, binds directly to intermediates of DNA replication, and may act as an engine that translocates the FA core complex along DNA [45]. Together with FANCD1/BRCA2 and FANCD2, the discovery of FANCJ/BRIP1 and FANCM/Hef provides the strongest evidence yet for a direct involvement of FA proteins in DNA repair functions associated with the resolution of DNA crosslinks and stalled replication forks.

Monoubiquitination is not the only modification sustained by the FANCD2 protein in response to DNA damage (fig. 4). FANCD2 is also one of the many targets of phosphorylation of the DNA damage sensors ATM and ATR [78, 79]. Phosphorylation of FANCD2 at Ser222 by ATM (cf. fig. 4) is necessary for the activation of the intra-S phase checkpoint [79, 80]. ATR mediated phosphorylation of FANCD2 in response to crosslinks or stalled replication forks depends on the intact FA core complex and the NBS1 protein [79]. These observations suggest that FANCD2 maintains genomic stability by the avoidance of cell cycle progression in situations of DNA replication stress due to damaged DNA and/or stalled replication forks [81]. Additionally, via the BRCA2 connection, FANCD2 is directly involved in recombinational DNA repair [13, 82]. A number of recent studies use the FA-deficient chicken cell line DT40 to investigate the role of FA genes in crosslink repair (e.g. [46, 47, 70, 83]). An important result of these studies is that the FA genes, in concert with other caretakers such as BLM, appear to promote homologous recombination, translesion synthesis and mutational repair of endogenously generated abasic sites. However, as with all non-human and transformed cell lines, caution is warranted in extrapolating data obtained from transformed cell lines. A case in point is the very popular, p53 deficient DT40 chicken cell line which, if FANCD2 deficient, shows a twofold elevation of spontaneous SCEs [83] which is not seen in primary human FANCD2 cells.

The FA-Cancer Connection

Severe anemia and pancytopenia due to bone marrow failure are life threatening clinical features of FA. Additionally, the disease is associated with a high risk of neoplasia, most frequently acute myeloid leukemia (AML) and squamous cell carcinoma, but many other types of neoplasia have been described [16, 21]. Inactivation of the FA/BRCA pathway might be an early step in developing sporadic malignancies, especially in cancer types that occur frequently in FA. Acquired dysfunction (deletion or reduced expression) of the *FANCA* gene has been described in patients with acute myelogenous leukemia [84, 85], and mutations of *FANCC* and *FANCG* have been found in a subset of pancreatic cancers [86]. Even though germline and somatic mutations in

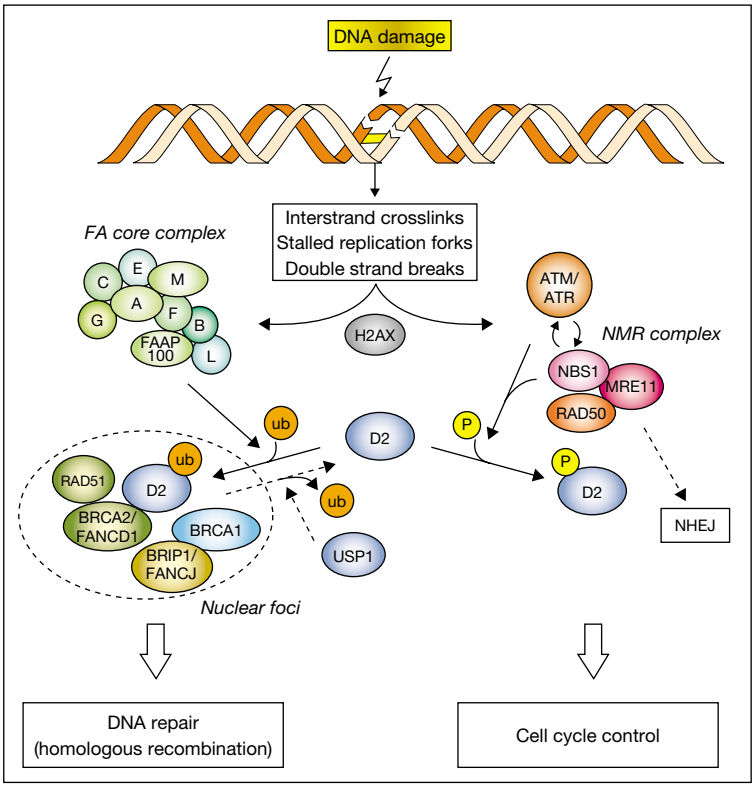

Fig. 4. Central role of the FANCD2 (D2) protein in the FA/BRCA network. In response to DNA-interstrand crosslinks, stalled replication forks and/or double strand breaks, the FA gene products form a nuclear complex consisting of at least eight separate proteins. The FANCL protein acts as the putative catalytic subunit of the core complex and monoubiquinates the FANCD2 protein at K561. The ubiquinated L-form of FANCD2 colocalizes in nuclear foci together with the products of the *BRCA1*, *FANCJ/BRIP1*, and *BRCA2*; *RAD51* genes that are known to contribute to homology directed DNA repair. Also shown is an alternative way of modification and activation of FANCD2 via phosphorylation by the DNA damage sensors ATM/ATR working together with the NBS1/MRE11/RAD50 protein complex. Phosphorylation of FANCD2 at S222 presumably leads to activation of an intra-S phase cell cycle checkpoint.

FANCC and *FANCG* may contribute to the occurrence of pancreatic cancer, the cancers with mutations in these FA genes arise in an apparent sporadic fashion rather as familiar cancer types. *FANCC* and *FANCG* mutations may therefore have a relatively low penetrance for the pancreatic cancer phenotype [87]. The most consistent dysfunction of FA genes found in a variety of human neoplasias involves the inactivation of the *FANCF* gene due to methylation of its CpG islands. Hypermethylation of *FANCF* has been observed in several kinds of

tumors, including cervical cancer [88], ovarian cancer [89, 90], AML [85], head and neck squamous cell carcinomas (HNSCC), non-small-cell lung cancers (NSCLC) [91], and a bladder carcinoma cell line [92]. The interpretation of these findings as to their causal links to tumor initiation remains tenuous, since *FANCF* is located on chromosome 11p15 within a hot-spot region for hypermethylation. It remains to be seen whether silencing of *FANCF* expression is a specific process or reflects the general changes in methylation pattern observed in many types of cancers and leukemias. Given the likely importance of the FA/BRCA pathway in the maintenance of genomic stability, it is rather surprising that apart from the above mentioned sporadic cases there appears to be no widespread involvement of the FA genes in tumorigenesis. This may indicate that functional FA genes are essential for the survival of malignant cells.

Fanconi Anemia Proteins: Players Within Networks

Even though the very existence of the FA core complex, its modification of FANCD2, and the subsequent formation of nuclear foci suggests a linear biological pathway leading from DNA damage to DNA repair, recent evidence favors the view that the FA proteins are part of an interchangeable network of multiple proteins involved in DNA replication, DNA recombination, and DNA repair [13, 93, 94]. For example, yeast two hybrid studies provide evidence for a consistent interaction between FA proteins and a number of proteins known to be involved in chromatin assembly and chromatin remodeling [95–97]. Changes in chromatin structure must precede any repair process in order to permit repair proteins to gain access to damaged DNA. Multisubunit protein complexes with partially overlapping and shared functions are required for the disruption and reformation of nucleosomal arrays during transcription, replication and repair [98]. Wild-type FANCC protein and the interacting protein FAZF have been shown to colocalize in nuclear repair foci and may be involved in transcriptional repression via chromatin remodeling [95]. Biochemical fractionation and immunocytochemical studies provide convincing evidence for a close physical association between FA proteins, nuclear matrix and interphase chromatin [65, 99, 100]. This association is enhanced by exposure to MMC but is abolished during the metaphase stage [101].

As shown in figure 5, the FA family of genes can be placed in the center of a network of genome maintenance genes [102] with close interconnections to the DNA damage sensors *ATM/ATR*, the NMR complex genes, the Werner and Bloom helicases and the *BRCA1* and *BRCA2* tumor suppressor genes [7, 103]. Moreover, interactions with Trp53 have been observed both in murine knockout and human FA-cell models [104–106]. As indicated by the shaded sector, biallelic mutations in *FANCD1/BRCA2* and *FANCJ/BRIP1* (a BRCA1 interacting protein) cause FA-like phenotypes which emphasizes the close relationship

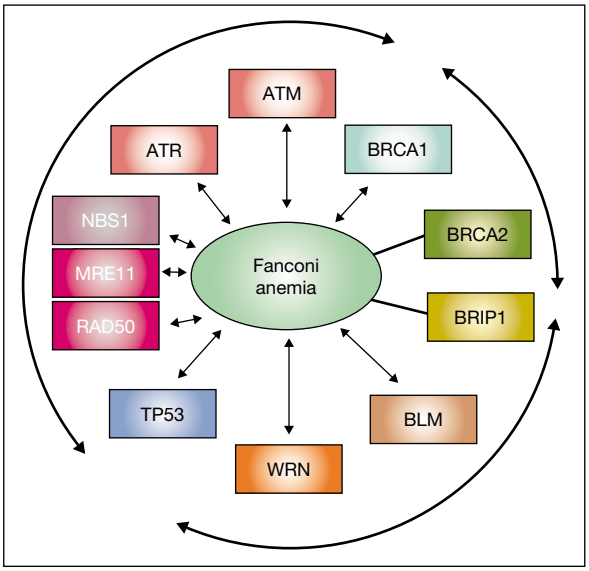

Fig. 5. Hypothetical concept of Fanconi anemia genes participating in a multifunctional network of caretaker genes, including the DNA-damage sensors ATM, ATR, NBS, MRE11, RAD50 and TP53, the DNA repair-helicases WRN, BLM and BACH1, and the cancer genes *BRCA1* and *BRCA2* involved in homology-directed DNA repair. The shaded sector emphasizes that biallelic mutations in BRCA2 and BACH1/BRIP1 cause a FA-like phenotype. Modified after Surralles et al. [7].

of these (downstream) genes with the FA core complex members. Since a number of FA patients have not been assigned to any of the known FA genes, it would come as no surprise if some of these unclassified FA patients would turn out to carry biallelic mutations in *BRCA1* itself, or in any of the numerous *BRCA1*-interacting genes [107]. In the context of the striking oxygen hypersensitivity phenotype of FA cells, it might be relevant that BRCA1 has been shown to induce antioxidant gene expression and resistance to oxidative stress [108]. Moreover, both BRCA1 and BRCA2 have been reported as instrumental in transcription coupled repair of oxidative DNA-lesions in human cells, even though this needs to be confirmed by additional studies [109].

Genetic Instability and Oxidative Stress: A Causal Connection?

One can hardly avoid the conclusion that FA cells lack sufficient protection against the damaging effects of reactive oxygen species [110–112]. Pagano and coworkers [111, 112] make a persuasive case that many of the clinical and cellular abnormalities observed in FA, including congenital malformations,

Table 4. The FA-oxygen sensitivity connection [111]

Oxidative stress parameters[a]	Function of FA proteins in redox-related pathways[a]
↑ 8-OHdG Luminol-dependent chemiluminescence SOD-sensitive clastogenic factor TNFα	A) FANCC: ↑ Gluthathione S-transferase (GSTP1) ↓ NADPH cytochrome P-450 reductase electron transport in microsomal membrane Transcriptional regulation of NFκB, COX2, HSP70
↓ Catalase activity Thioredoxin expression Cell growth in O_2 rich atmosphere Oxidative stress-related cytoskeleton abnormalities Iron-sensitive G_2 arrest	B) FANCG: ↑ CYP2E1 8-OHdG after H_2O_2 or MMC exposure

[a]↑ increase; ↓ decrease.

pigmentation changes, bone marrow failure and insulin resistance, may result from macromolecular damage caused by elevated levels of oxidative stress (cf. table 4). As early as in 1981, Joenje and coworkers detected a striking correlation between rates of chromosome breakage and oxygen tension in FA cells [29]. A subsequent study showed that growth and cloning efficiency of FA fibroblasts is severely impaired under ambient oxygen (20% v/v), but turns to near normal under hypoxic (5% v/v) cell culture conditions [38]. Saito and coworkers confirmed these observations and concluded that hypersensitivity towards oxygen is a 'uniform but secondary defect' in FA-cells which might, however, contribute to bone marrow failure [113]. At least in the murine *Fancc* knock-out model there is evidence for enhanced oxidant-mediated apoptosis of FA hematopoietic stem or progenitor cells [114], and repeated cycles of hypoxia-reoxygenation induce premature senescence in FA hematopoietic cells [115]. Testing the cell cycle behavior of FA-lymphoid cell lines under various oxygen tensions, Poot et al. suggested that oxygen sensitivity in FA cells might be mediated by the amount of iron in the culture media, implicating a reduced capacity of FA cells to deal with (reactive oxygen) products of Fenton-type reactions [116]. Evidence for mutagenic activity of oxygen in FA cells was provided by passaging indicator plasmids through FA cells grown at different oxygen tensions [117]. The proponents of the 'oxidative stress theory in FA' believe that oxidative stress characterizes the metabolic situation of several types of patient blood cells, both in vitro and in vivo [111, 118]. Overexpression of the antioxidant thioredoxin apparently mitigates the clastogenic effects of MMC or DEB in FA fibroblasts [119]. A similar effect has previously been reported with

DL-alpha-tocopherol (a potent lipophilic antioxidant) [120]. The hypersensitivity of FA cells towards MMC or DEB may be caused by the property of these agents (that need to be metabolically activated) to generate oxygen radicals under in vitro conditions [121, 122]. Several studies have implicated the *FANCC* gene in antioxidant defence functions [111, 123], and specifically in the repair of oxidatively damaged DNA [124]. A direct role of FANCC in the protection against oxidative damage is supported by a murine model in which both the genes for Cu/Zn superoxide dismutase and *Fancc* have been inactivated [125]. In addition to liver abnormalities, and in contrast to single FA gene knock-outs, these mice exhibit the FA phenotype of impaired hematopoiesis as if inactivation of Fancc in the absence of the scavenger SOD would lead to bone marrow failure, possibly due to the overwhelming effects of endogenously generated reactive oxygen species.

If FA genes play a major role in the defense against endogenous oxidative damage, one should expect to find increased baseline levels of oxygen-related DNA base modifications such as 8-OHdG in FA patients. However, such evidence is lacking [126, 127] or controversial [128, 129]. What remains unclear at this point is whether the in vitro hypersensitivity toward oxygen causes greater accumulations of DNA lesions in FA as opposed to non-FA cells, or whether the removal of oxidative lesions is less effective or somehow impaired in cells which carry mutations in one of the FA genes. There is compelling evidence, however, that oxidative stress and/or oxidative damage activates the FA/BRCA pathway by multimerization and interaction of FANCA, FANCG and FANCC, transport into the nucleus, and FA core complex formation [68]. An attractive hypothesis posits that FA proteins act as sensors of the cellular redox status and translate this information to other members of the caretaker gene network [13]. It is quite obvious that warm-blooded and long-lived mammals that generate high levels of reactive oxygen species during normal cellular functions (i.e. oxidative phosphorylation) require special protection against the 'time bomb' within [130]. Although oxidized bases, AP sites and DNA single-strand breaks are removed by DNA excision repair [131], the evolutionarily recent family of FA proteins might serve the purpose of removal, via recombinational repair, of double strand breaks as the most deleterious end-products of oxidative DNA damage. With defective FA genes cells suffer the consequences of oxidative stress without sufficient protection, manifesting as chromosomal instability and early neoplasia.

Somatic Reversion as a (Positive) Consequence of Genetic Instability

In recessive diseases, somatic reversion of one of the two inherited mutations restores heterozygosity in the descendants of the reverted cell [14, 132]. Depending on the mechanism of somatic reversion, the function of the affected cell lineage may be partly or completely restored. Complete restoration of a

cellular phenotype to wildtype usually results from mechanisms such as intragenic recombination, back-mutation (reverse point mutation), or gene conversion [14, 133]. Partial restoration of protein function has been observed with so-called compensating or second site mutations [134]. Such second site mutations in *cis* leave the constitutional mutation unchanged but alter the downstream DNA sequence via insertion, deletion or point mutation. As a consequence of the compensating mutation a protein with at least partial function is produced [134].

Revertant mosaicism is a rather frequent phenomenon in the autosomal recessive genetic instability syndromes, particularly Bloom syndrome and FA [24, 25, 133, 135, 136]. In FA, intragenic recombination, gene conversion and compensatory second site mutations have been reported in lymphoblastoid cell lines of four FANCC patients, and cytogenetic evidence for mosaicism in additional five unclassified patients [24, 134]. Five compound heterozygous FANCA patients have been reported to date who developed mosaicism as a consequence of a putative back mutation or gene conversion [25, 133]. In addition to FA-C and FA-A patients, four FANCD2 patients were reported as mosaics [137], proving that somatic reversion also affects the central *FANCD2* gene.

Mechanisms of Somatic Reversion Observed in FA Patients

Two FANCA patients with homozygous mutations were shown to harbor compensatory mutations in their peripheral blood mononuclear cells [134]. An unusual compensatory mutation involving the loss of the natural splice acceptor of exon 7 and the use of the next 3′AG for splicing leading to restoration of the open reading frame was found in a heterozygous FANCL patient (Kalb et al., manuscript in preparation). In addition, the case history of a patient reported by Gross et al. demonstrates that compensatory second site mutations can also arise during the in vitro cultivation of lymphoblastoid cells, explaining the conversion of these cells from MMC-sensitivity to MMC-resistance [25]. Intragenic crossover represents a reversion mechanism that requires compound heterozygosity with mutually distant locations of the paternal and the maternal mutations. It has been described as the predominant mechanism of reversion in Bloom syndrome [136] and in the lymphoblastoid cell line of occasional FANCC patients [24]. Gene conversion or back mutation was postulated as the mechanism of reversion in four FA-A compound heterozygous patients described by Gross et al. [25]. In each of these patients, reversion led to the restoration of precisely the wildtype sequence. How can this apparent non-randomness be reconciled with the stochastic nature of mutations? One obvious explanation would be that selection creates a proliferative advantage for cells with complete rather than only partial restoration of protein function. In addition, there might be constraints imposed by DNA structure which would favor the restoration of the original sequence. Back mutation combined

with selection therefore might explain the surprisingly uniform pattern of reversion in the four reported mosaic FA-A patients. Another possibility would be gene conversion. Gene conversion requires an intact DNA strand opposite to the mutation, a condition which is fulfilled during the postreplicative phase of the cell cycle when sister chromatids are paired. However, gene conversion through sister chromatid pairing does not work with constitutional mutations since both sister chromatids carry the respective mutation. In this situation, gene conversion requires some sort of somatic pairing between homologous chromosomes. Since the *FANCA* gene is located on human chromosome 16 that harbors a large block of heterochromatin known to promote somatic pairing [138], gene conversion via somatic pairing might occur in FA-A patients who are compound heterozygotes for point mutations or small deletions. It would not, however, work in cases where the second allele involves a large deletion.

The clinical course of FA is highly variable and may be determined in part by complementation group and mutation type [139, 140]. Whether revertant mosaicism leads to clinical improvement depends on when and where the reversion occurred during evolution of the various bone marrow cell lineages [24, 133]. The best prospects for clinical improvement probably exist if the reversion takes place in a hematopoietic stem cell as evidenced by reversal of the FA phenotype in all descendant blood cell lineages [133, 141]. Regarding the prospects of future gene therapy, the evidence for in vivo selective advantage of spontaneously reverted stem cell progeny in mosaic patients is encouraging indeed. At the same time, like few other examples, this phenomenon illustrates the interplay between genomic instability and cellular selection which may be at the root of cancer and aging.

References

1 Hasty P, Campisi J, Hoeijmakers J, van Steeg H, Vijg J: Aging and genome maintenance: lessons from the mouse? Science 2003;299:1355–1359.
2 Hoeijmakers JH: Genome maintenance mechanisms for preventing cancer. Nature 2001;411:366–374.
3 Furuichi Y: Premature aging and predisposition to cancers caused by mutations in RecQ family helicases. Ann NY Acad Sci 2001;928:121–131.
4 Campisi J: Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 2005;120:513–522.
5 Prime SS, Thakker NS, Pring M, Guest PG, Paterson IC: A review of inherited cancer syndromes and their relevance to oral squamous cell carcinoma. Oral Oncol 2001;37:1–16.
6 Kipling D, Davis T, Ostler EL, Faragher RG: What can progeroid syndromes tell us about human aging? Science 2004;305:1426–1431.
7 Surralles J, Jackson SP, Jasin M, Kastan MB, West SC, Joenje H: Molecular cross-talk among chromosome fragility syndromes. Genes Dev 2004;18:1359–1370.
8 Marrone A, Dokal I: Dyskeratosis congenita: molecular insights into telomerase function, ageing and cancer. Expert Rev Mol Med 2004;6:1–23.

9 Burke B, Stewart CL: Life at the edge: the nuclear envelope and human disease. Nat Rev Mol Cell Biol 2002;3:575–585.
10 Joenje H, Patel KJ: The emerging genetic and molecular basis of Fanconi anaemia. Nat Rev Genet 2001;2:446–457.
11 Ahmad SI, Hanaoka F, Kirk SH: Molecular biology of Fanconi anemia – an old problem, a new insight. Bioessays 2002;24:439–448.
12 Tischkowitz M, Dokal I: Fanconi anaemia and leukaemia – clinical and molecular aspects. Br J Haematol, 2004;126:176–191.
13 Thompson LH, Hinz JM, Yamada NA, Jones NJ: How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen 2005;45:128–142.
14 Hirschhorn R: In vivo reversion to normal of inherited mutations in humans. J Med Genet 2003;40:721–728.
15 Schroeder-Kurth TM, Auerbach AD, Obe G: Fanconi anemia. Clinical, cytogenetic and experimental aspects. Springer-Verlag, Berlin, Germany, 1989.
16 Alter BP: Cancer in Fanconi anemia, 1927–2001. Cancer 2003;97:425–440.
17 Verlander PC, Kaporis A, Liu Q, Zhang Q, Seligsohn U, Auerbach AD: Carrier frequency of the IVS4 + 4 A→T mutation of the Fanconi anemia gene *FAC* in the Ashkenazi Jewish population. Blood 1995;86:4034–4038.
18 Callen E, Casado JA, Tischkowitz MD, Bueren JA, Creus A, Marcos R, Dasi A, Estella JM, et al: A common founder mutation in *FANCA* underlies the world's highest prevalence of Fanconi anemia in Gypsy families from Spain. Blood 2005;105:1946–1949.
19 Kutler DI, Auerbach AD: Fanconi anemia in Ashkenazi Jews. Fam Cancer 2004;3:241–248.
20 Kwee ML, van der Kleij JM, van Essen AJ, Begeer JH, Joenje H, Arwert F, ten Kate LP: An atypical case of Fanconi anemia in elderly sibs. Am J Med Genet 1997;68:362–366.
21 Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, Giampietro PF, Hanenberg H, Auerbach AD: A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood 2003;101:1249–1256.
22 Wajnrajch MP, Gertner JM, Huma Z, Popovic J, Lin K, Verlander PC, Batish SD, Giampietro PF, et al: Evaluation of growth and hormonal status in patients referred to the International Fanconi Anemia Registry. Pediatrics 2001;107:744–754.
23 Rosenberg PS, Greene MH, Alter BP: Cancer incidence in persons with Fanconi anemia. Blood 2003;101:822–826.
24 Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, van Weel M, Pauli RM, Shahidi NT, et al: Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet 1997;5:137–148.
25 Gross M, Hanenberg H, Lobitz S, Friedl R, Herterich S, Dietrich R, Gruhn B, Schindler D, Hoehn H: Revertant mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. Cytogenet Genome Res 2002;98:126–135.
26 Liu JM, Auerbach AD, Young NS: Fanconi anemia presenting unexpectedly in an adult kindred with no dysmorphic features. Am J Med 1991;91:555–557.
27 Rosenberg PS, Socie G, Alter BP, Gluckman E: Risk of head and neck squamous cell cancer and death in patients with Fanconi anemia who did and did not receive transplants. Blood 2005;105:67–73.
28 Schroeder TM, Anschütz F, Knopp A: Spontane Chromosomenaberrationen bei familiärer Panmyelopathie. Humangenetik 1964;1:194–196.
29 Joenje H, Arwert F, Eriksson AW, de Koning H, Oostra AB: Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. Nature 1981;290:142–143.
30 Evdokimova VN, McLoughlin RK, Wenger SL, Grant SG: Use of the glycophorin A somatic mutation assay for rapid, unambiguous identification of Fanconi anemia homozygotes regardless of GPA genotype. Am J Med Genet A 2005;135:59–65.
31 Sasaki MS, Tonomura A: A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res 1973;33:1829–1836.
32 Auerbach AD, Wolman SR: Carcinogen-induced chromosome breakage in Fanconi's anaemia heterozygous cells. Nature 1978;271:69–71.
33 Dutrillaux B, Aurias A, Dutrillaux AM, Buriot D, Prieur M: The cell cycle of lymphocytes in Fanconi anemia. Hum Genet 1982;62:327–332.

34 Kubbies M, Schindler D, Hoehn H, Schinzel A, Rabinovitch PS: Endogenous blockage and delay of the chromosome cycle despite normal recruitment and growth phase explain poor proliferation and frequent endomitosis in Fanconi anemia cells. Am J Hum Genet 1985;37:1022–1030.
35 Seyschab H, Sun Y, Friedl R, Schindler D, Hoehn H: G2 phase cell cycle disturbance as a manifestation of genetic cell damage. Hum Genet 1993;92:61–68.
36 Akkari YM, Bateman RL, Reifsteck CA, D'Andrea AD, Olson SB, Grompe M: The 4N cell cycle delay in Fanconi anemia reflects growth arrest in late S phase. Mol Genet Metabol 2001;74:403–412.
37 Seyschab H, Friedl R, Sun Y, Schindler D, Hoehn H, Hentze S, Schroeder-Kurth T: Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood 1995;85:2233–2237.
38 Schindler D, Hoehn H: Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet 1988;43:429–435.
39 Kalb R, Duerr M, Wagner M, Herterich S, Gross M, Digweed M, Joenje H, Hoehn H, Schindler D: Lack of sensitivity of primary Fanconi's anemia fibroblasts to UV and ionizing radiation. Radiat Res 2004;161:318–325.
40 Djuzenova CS, Rothfuss A, Oppitz U, Speit G, Schindler D, Hoehn H, Flentje M: Response to X-irradiation of Fanconi anemia homozygous and heterozygous cells assessed by the single-cell gel electrophoresis (comet) assay. Lab Invest 2001;81:185–192.
41 Levitus M, Rooimans MA, Steltenpool J, Cool NF, Oostra AB, Mathew CG, Hoatlin ME, Waisfisz Q, et al: Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. Blood 2004;103: 2498–2503.
42 Rahman N, Ashworth A: A new gene on the X involved in Fanconi anemia. Nat Genet 2004;36: 1142–1143.
43 Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, Rio P, Batish SD, Kalb R, et al: The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet 2005;37:931–933.
44 Levitus M, Waisfisz Q, Godthelp BC, Vries YD, Hussain S, Wiegant WW, Elghalbzouri-Maghrani E, Steltenpool J, et al: The DNA helicase BRIP1 is defective in Fanconi anemia complementation group. J Nat Genet 2005;37:934–935.
45 Meetei AR, Medhurst AL, Ling C, Xue Y, Singh TR, Bier P, Steltenpool J, Stone S, et al: A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet 2005;37:958–963.
46 Mosedale G, Niedzwiedz W, Alpi A, Perrina F, Pereira-Leal JB, Johnson M, Langevin F, Pace P, Patel KJ: The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. Nat Struct Mol Biol 2005;12:763–771.
47 Bridge WL, Vandenberg CJ, Franklin RJ, Hiom K: The BRIP1 helicase functions independently of BRCA1 in the Fanconi anemia pathway for crosslink repair. Nat Genet 2005;37:953–957.
48 Hirsch B, Shimamura A, Moreau L, Baldinger S, Hagalshiekh M, Bostrom B, Sencer S, D'Andrea AD: Association of biallelic *BRCA2*/*FANCD1* mutations with spontaneous chromosomal instability and solid tumors of childhood. Blood 2004;103:2554–2559.
49 Wagner JE, Tolar J, Levran O, Scholl T, Deffenbaugh A, Satagopan J, Ben-Porat L, Mah K, et al: Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. Blood 2004;103:3226–3229.
50 Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, De Die-Smulders C, Persky N, Grompe M, et al: Biallelic inactivation of *BRCA2* in Fanconi anemia. Science 2002;297:606–609.
51 Popp H, Kalb R, Fischer A, Lobitz S, Kokemohr I, Hanenberg H, Schindler D: Screening Fanconi anemia lymphoid cell lines of non-A, C, D2, E, F, G subtypes for defects in BRCA2/FANCD1. Cytogenet Genome Res 2003;103:54–57.
52 Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, Leurs C, Cooper RJ, Gottsche K, et al: Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 2002;30:410–420.
53 Chandra S, Levran O, Jurickova I, Maas C, Kapur R, Schindler D, Henry R, Milton K, et al: A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther 2005;12:976–984.
54 Meetei AR, Yan Z, Wang W: FANCL replaces BRCA1 as the likely ubiquitin ligase responsible for FANCD2 monoubiquination. Cell Cycle 2004;3:179–181.

55 Meetei AR, de Winter JP, Medhurst AL, Wallisch M, Waisfisz Q, van de Vrugt HJ, Oostra AB, Yan Z, et al: A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet 2003;35:165–170.
56 Holzel M, van Diest PJ, Bier P, Wallisch M, Hoatlin ME, Joenje H, de Winter JP: FANCD2 protein is expressed in proliferating cells of human tissues that are cancer-prone in Fanconi anaemia. J Pathol 2003;201:198–203.
57 Nadler JJ, Braun RE: Fanconi anemia complemenation group C is required for proliferation of murine primordial germ cells. Genesis 2000;27:117–123.
58 Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, D'Andrea AD: Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 2001;7:249–262.
59 Freie B, Ciccone SL, Li X, Plett PA, Orschell CM, Srour EF, Hanenberg H, Schindler D, Lee SH, Clapp DW: A role for the Fanconi anemia C protein in maintaining the DNA damage-induced G2 checkpoint. J Biol Chem 2004;279:50986–50993.
60 Zanier R, Briot D, Dugas du Villard JA, Sarasin A, Rosselli F: Fanconi anemia C gene product regulates expression of genes involved in differentiation and inflammation. Oncogene 2004;23: 5004–5013.
61 Garcia-Higuera I, Kuang Y, Naf D, Wasik J, D'Andrea AD: Fanconi anemia proteins FANCA, FANCC, and FANCG/XRCC9 interact in a functional nuclear complex. Mol Cell Biol 1999;19: 4866–4873.
62 Gordon SM, Buchwald M: Fanconi anemia protein complex: mapping protein interactions in the yeast 2- and 3-hybrid systems. Blood 2003;102:136–141.
63 Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, Bruun D, Thayer M, Cox B, et al: Positional cloning of a novel Fanconi anemia gene, *FANCD2*. Mol Cell 2001;7:241–248.
64 Medhurst AL, Huber PA, Waisfisz Q, de Winter JP, Mathew CG: Direct interactions of the five known Fanconi anaemia proteins suggest a common functional pathway. Hum Mol Genet 2001;10:423–429.
65 Thomashevski A, High AA, Drozd M, Shabanowitz J, Hunt DF, Grant PA, Kupfer GM: The Fanconi anemia core complex forms four complexes of different sizes in different subcellular compartments. J Biol Chem 2004;279:26201–26209.
66 Park S-J, Ciccone SLM, Beck BD, Hwang B, Freie B, Clapp DW, Lee S-H: Oxidative stress/damage induces multimerization and interaction of Fanconi anemia proteins. J Biol Chem 2004;279: 30053–30059.
67 Shimamura A, de Oca RM, Svenson JL, Haining N, Moreau LA, Nathan DG, D'Andrea AD: A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood 2002;100:4649–4654.
68 Digweed M, Rothe S, Demuth I, Scholz R, Schindler D, Stumm M, Grompe M, Jordan A, Sperling K: Attenuation of the formation of DNA-repair foci containing RAD51 in Fanconi anaemia. Carcinogenesis 2002;23:1121–1126.
69 Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, Wang ZQ, Jasin M: Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci USA 2005;102:1110–1115.
70 Niedzwiedz W, Mosedale G, Johnson M, Ong CY, Pace P, Patel KJ: The Fanconi anaemia gene *FANCC* promotes homologous recombination and error-prone DNA repair. Mol Cell 2004;15:607–620.
71 Castillo V, Cabre O, Marcos R, Surralles J: Molecular cloning of the *Drosophila* Fanconi anaemia gene FANCD2 cDNA. DNA Repair (Amst) 2003;2:751–758.
72 Liu TX, Howlett NG, Deng M, Langenau DM, Rhodes J, Kanki JP, D'Andrea AD, Look AT: Knockdown of zebrafish Fancd2 causes developmental abnormalities via p53-dependent apoptosis. Dev Cell 2003;5:903–914.
73 Nijman SM, Huang TT, Dirac AM, Brummelkamp TR, Kerkhoven RM, D'Andrea AD, Bernards R: The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol Cell 2005;17:331–339.
74 Dequen F, St-Laurent JF, Gagnon SN, Carreau M, Desnovers S: The *Caenorhabditis elegans FancD2* ortholog is required for survival following DNA damage. Comp Biochem Physiol B Biochem Mol Biol 2005;141:453–460.
75 Kalb R, Neveling K, Hoehn H, Joenje H, Surrales J, Auerbach AD, Hanenberg H, Schindler D: Hypomorphic mutations in the *FANCD2* gene are compatible with survival but cause a severe Fanconi anemia phenotype; submitted 2005.

76 Lomonosov M, Anand S, Sangrithi M, Davies R, Venkitaraman AR: Stabilization of stalled DNA replication forks by the BRCA2 breast cancer susceptibility protein. Genes Dev 2003;17: 3017–3022.
77 Park WH, Margossian S, Horwitz AA, Simons AM, D'Andrea AD, Parvin JD: Direct DNA binding activity of the fanconi anemia D2 protein. J Biol Chem 2005;280:23593–23598.
78 Andreassen PR, D'Andrea AD, Taniguchi T: ATR couples FANCD2 monoubiquitination to the DNA-damage response. Genes Dev 2004;18:1958–1963.
79 Pichierri P, Rosselli F: The DNA crosslink-induced S-phase checkpoint depends on ATR-CHK1 and ATR-NBS1-FANCD2 pathways. EMBO J 2004;23:1178–1187.
80 Taniguchi T, Garcia-Higuera I, Andreassen PR, Gregory RC, Grompe M, D'Andrea AD: S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood 2002;100:2414–2420.
81 Howlett NG, Taniguchi T, Durkin SG, D'Andrea AD, Glover TW: The Fanconi anemia pathway is required for the DNA replication stress response and for the regulation of common fragile site stability. Hum Mol Genet 2005;14:693–701.
82 Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, Kim ST, Lane WS, Kastan MB, D'Andrea AD: Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell 2002;109:459–472.
83 Hirano S, Yamamoto K, Ishiai M, Yamazoe M, Seki M, Matsushita N, Ohzeki M, Yamashita YM, et al: Functional relationships of FANCC to homologous recombination, translesion synthesis, and BLM. EMBO J 2005;24:418–427.
84 Lensch MW, Tischkowitz M, Christianson TA, Reifsteck CA, Speckhart SA, Jakobs PM, O'Dwyer ME, Olson SB, et al: Acquired FANCA dysfunction and cytogenetic instability in adult acute myelogenous leukemia. Blood 2003;102:7–16.
85 Tischkowitz M, Ameziane N, Waisfisz Q, De Winter JP, Harris R, Taniguchi T, D'Andrea A, Hodgson SV, Mathew CG, Joenje H: Bi-allelic silencing of the Fanconi anaemia gene *FANCF* in acute myeloid leukaemia. Br J Haematol 2003;123:469–471.
86 van der Heijden MS, Yeo CJ, Hruban RH, Kern SE: Fanconi anemia gene mutations in young-onset pancreatic cancer. Cancer Res 2003;63:2585–2588.
87 Rogers CD, van der Heijden MS, Brune K, Yeo CJ, Hruban RH, Kern SE, Goggins M: The genetics of *FANCC* and *FANCG* in familial pancreatic cancer. Cancer Biol Ther 2004;3:167–169.
88 Narayan G, Arias-Pulido H, Nandula SV, Basso K, Sugirtharaj DD, Vargas H, Mansukhani M, Villella J, et al: Promoter hypermethylation of *FANCF*: disruption of Fanconi Anemia-BRCA pathway in cervical cancer. Cancer Res 2004;64:2994–2997.
89 Taniguchi T, Tischkowitz M, Ameziane N, Hodgson SV, Mathew CG, Joenje H, Mok SC, D'Andrea AD: Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med 2003;9:568–574.
90 Dhillon VS, Shahid M, Husain SA: CpG methylation of the *FHIT*, *FANCF*, *cyclin-D2*, *BRCA2* and *RUNX3* genes in Granulosa cell tumors (GCTs) of ovarian origin. Mol Cancer 2004;3:33.
91 Marsit CJ, Liu M, Nelson HH, Posner M, Suzuki M, Kelsey KT: Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. Oncogene 2004;23:1000–1004.
92 Neveling K, Kalb R, Friedl R, Florl A, Harder C, Herterich S, Nanda I, Hoehn H, et al: Disruption of the FA/BRCA pathway due to hypermethylation of the Fanconi anemia gene *FANCF*: a rare event in bladder carcinoma; submitted 2005.
93 Venkitaraman AR: Tracing the network connecting BRCA and Fanconi anaemia proteins. Nat Rev Cancer 2004;4:266–276.
94 Macé G, Bogliolo M, Guervilly J-H, Dugas du Villard JA, Rosselli F: 3R coordination by Fanconi anemia proteins. Biochimie 2005;2410:647–658.
95 Hoatlin ME, Zhi Y, Ball H, Silvey K, Melnick A, Stone S, Arai S, Hawe N, Owen G, Zelent A, Licht JD: A novel BTB/POZ transcriptional repressor protein interacts with the Fanconi anemia group C protein and PLZF. Blood 1999;94:3737–3747.
96 Otsuki T, Furukawa Y, Ikeda K, Endo H, Yamashita T, Shinohara A, Iwamatsu A, Ozawa K, Liu JM: Fanconi anemia protein, FANCA, associates with BRG1, a component of the human SWI/SNF complex. Hum Mol Genet 2001;10:2651–2660.

97 Reuter TY, Medhurst AL, Waisfisz Q, Zhi Y, Herterich S, Hoehn H, Gross HJ, Joenje H, et al: Yeast two-hybrid screens imply involvement of Fanconi anemia proteins in transcription regulation, cell signaling, oxidative metabolism, and cellular transport. Exp Cell Res 2003;289:211–221.
98 Davie JR, Moniwa M: Control of chromatin remodeling. Crit Rev Eukaryot Gene Expr 2000;10: 303–325.
99 Kupfer GM, Naf D, Suliman A, Pulsipher M, D'Andrea AD: The Fanconi anaemia proteins, FAA and FAC, interact to form a nuclear complex. Nat Genet 1997;17:487–490.
100 Qiao F, Moss A, Kupfer GM: Fanconi anemia proteins localize to chromatin and the nuclear matrix in a DNA damage- and cell cycle-regulated manner. J Biol Chem 2001;276:23391–23396.
101 Mi J, Kupfer GM: The Fanconi anemia core complex associates with chromatin during S phase. Blood 2005;105:759–766.
102 Begley TJ, Samson LD: Network responses to DNA damaging agents. DNA Repair (Amst) 2004;3:1123–1132.
103 Pichierri P, Rosselli F: Fanconi anemia proteins and the S phase checkpoint. Cell Cycle 2004;3: 698–700.
104 Liebetrau W, Budde A, Savoia A, Grummt F, Hoehn H: p53 activates Fanconi anemia group C gene expression. Hum Mol Genet 1997;6:277–283.
105 Freie B, Li X, Ciccone SL, Nawa K, Cooper S, Vogelweid C, Schantz L, Haneline LS, et al: Fanconi anemia type C and p53 cooperate in apoptosis and tumorigenesis. Blood 2003;102: 4146–4152.
106 Houghtaling S, Newell A, Akkari Y, Taniguchi T, Olson S, Grompe M: Fancd2 functions in a double strand break repair pathway that is distinct from non-homologous end joining. Hum Mol Genet 2005;14:3027–3033.
107 Liu Y, West SC: Distinct functions of BRCA1 and BRCA2 in double-strand break repair. Breast Cancer Res 2002;4:9–13.
108 Bae I, Fan S, Meng Q, Rih JK, Kim HJ, Kang HJ, Xu J, Goldberg ID, Jaiswal AK, Rosen EM: BRCA1 induces antioxidant gene expression and resistance to oxidative stress. Cancer Res 2004;64:7893–7909.
109 Le Page F, Randrianarison V, Marot D, Cabannes J, Perricaudet M, Feunteun J, Sarasin A: BRCA1 and BRCA2 are necessary for the transcription-coupled repair of the oxidative 8-oxoguanine lesion in human cells. Cancer Res 2000;60:5548–5552.
110 Liebetrau W, Runger TM, Baumer A, Henning C, Gross O, Schindler D, Poot M, Hoehn H: Exploring the role of oxygen in Fanconi's anemia. Recent Results Cancer Res 1997;143: 353–367.
111 Pagano G, Youssoufian H: Fanconi anaemia proteins: major roles in cell protection against oxidative damage. Bioessays 2003;25:589–595.
112 Pagano G, Degan P, d'Ischia M, Kelly FJ, Nobili B, Pallardo FV, Youssoufian H, Zatterale A: Oxidative stress as a multiple effector in Fanconi anaemia clinical phenotype. Eur J Haematol 2005;75:93–100.
113 Saito H, Hammond AT, Moses RE: Hypersensitivity to oxygen is a uniform and secondary defect in Fanconi anemia cells. Mutat Res 1993;294:255–262.
114 Saadatzadeh MR, Bijangi-Vishehsaraei K, Hong P, Bergmann H, Haneline LS: Oxidant hypersensitivity of Fanconi anemia type C-deficient cells is dependent on a redox-regulated apoptotic pathway. J Biol Chem 2004;279:16805–16812.
115 Zhang X, Li J, Sejas DP, Pang Q: Hypoxia-reoxygenation induces premature senescence in FA bone marrow hematopoietic cells. Blood 2005;106:75–85.
116 Poot M, Gross O, Epe B, Pflaum M, Hoehn H: Cell cycle defect in connection with oxygen and iron sensitivity in Fanconi anemia lymphoblastoid cell lines. Exp Cell Res 1996;222:262–268.
117 Liebetrau W, Runger TM, Mehling BE, Poot M, Hoehn H: Mutagenic activity of ambient oxygen and mitomycin C in Fanconi's anaemia cells. Mutagenesis 1997;12:69–77.
118 Pagano G, Korkina LG: Prospects for nutritional interventions in the clinical management of Fanconi anemia. Cancer Causes Control 2000;11:881–889.
119 Ruppitsch W, Meisslitzer C, Hirsch-Kauffmann M, Schweiger M: Overexpression of thioredoxin in Fanconi anemia fibroblasts prevents the cytotoxic and DNA damaging effect of mitomycin C and diepoxybutane. FEBS Lett 1998;422:99–102.

120 Pincheira J, Bravo M, Santos MJ, de la Torre C, Lopez-Saez JF: Fanconi anemia lymphocytes: effect of DL-alpha-tocopherol (Vitamin E) on chromatid breaks and on G2 repair efficiency. Mutat Res 2001;461:265–271.
121 Clarke AA, Philpott NJ, Gordon-Smith EC, Rutherford TR: The sensitivity of Fanconi anaemia group C cells to apoptosis induced by mitomycin C is due to oxygen radical generation, not DNA crosslinking. Br J Haematol 1997;96:240–247.
122 Pagano G: Redox-modulated xenobiotic action and ROS formation: a mirror or a window? Hum Exp Toxicol 2002;21:77–81.
123 Pagano G: Mitomycin C and diepoxybutane action mechanisms and FANCC protein functions: further insights into the role for oxidative stress in Fanconi's anaemia phenotype. Carcinogenesis 2000;21:1067–1068.
124 Lackinger D, Ruppitsch W, Ramirez MH, Hirsch-Kauffmann M, Schweiger M: Involvement of the Fanconi anemia protein FA-C in repair processes of oxidative DNA damages. FEBS Lett 1998;440:103–106.
125 Hadjur S, Ung K, Wadsworth L, Dimmick J, Rajcan-Separovic E, Scott RW, Buchwald M, Jirik FR: Defective hematopoiesis and hepatic steatosis in mice with combined deficiencies of the genes encoding Fancc and Cu/Zn superoxide dismutase. Blood 2001;98:1003–1011.
126 Will O, Schindler D, Boiteux S, Epe B: Fanconi's anaemia cells have normal steady-state levels and repair of oxidative DNA base modifications sensitive to Fpg protein. Mutat Res 1998;409: 65–72.
127 Zunino A, Degan P, Vigo T, Abbondandolo A: Hydrogen peroxide: effects on DNA, chromosomes, cell cycle and apoptosis induction in Fanconi's anemia cell lines. Mutagenesis 2001;16:283–288.
128 Degan P, Bonassi S, De Caterina M, Korkina LG, Pinto L, Scopacasa F, Zatterale A, Calzone R, Pagano G: In vivo accumulation of 8-hydroxy-2′-deoxyguanosine in DNA correlates with release of reactive oxygen species in Fanconi's anaemia families. Carcinogenesis 1995;16: 735–741.
129 Pagano G, Degan P, d'Ischia M, Kelly FJ, Pallardo FV, Zatterale A, Anak SS, Akisik EE, et al: Gender- and age-related distinctions for the in vivo prooxidant state in Fanconi anaemia patients. Carcinogenesis 2004;25:1899–1909.
130 Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, et al: Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 2005;308:1909–1911.
131 Barzilai A, Yamamoto K: DNA damage responses to oxidative stress. DNA Repair (Amst) 2004;3:1109–1115.
132 Youssoufian H, Pyeritz RE: Mechanisms and consequences of somatic mosaicism in humans. Nat Rev Genet 2002;3:748–758.
133 Gregory JJ Jr, Wagner JE, Verlander PC, Levran O, Batish SD, Eide CR, Steffenhagen A, Hirsch B, Auerbach AD: Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. Proc Natl Acad Sci USA 2001;98:2532–2537.
134 Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, Hoatlin ME, Ianzano L, Gibson RA, et al: Spontaneous functional correction of homozygous fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet 1999;22:379–383.
135 Ellis NA, Ciocci S, German J: Back mutation can produce phenotype reversion in Bloom syndrome somatic cells. Hum Genet 2001;108:167–173.
136 Ellis NA, Lennon DJ, Proytcheva M, Alhadeff B, Henderson EE, German J: Somatic intragenic recombination within the mutated locus BLM can correct the high sister-chromatid exchange phenotype of Bloom syndrome cells. Am J Hum Genet 1995;57:1019–1027.
137 Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, Guardiola P, Esperou H, Ferry C, et al: Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood 2005;105:1329–1336.
138 Haaf T, Steinlein K, Schmid M: Preferential somatic pairing between homologous heterochromatic regions of human chromosomes. Am J Hum Genet 1986;38:319–329.
139 Gillio AP, Verlander PC, Batish SD, Giampietro PF, Auerbach AD: Phenotypic consequences of mutations in the Fanconi anemia FAC gene: an International Fanconi Anemia Registry study. Blood 1997;90:105–110.

140 Faivre L, Guardiola P, Lewis C, Dokal I, Ebell W, Zatterale A, Altay C, Poole J, et al: Association of complementation group and mutation type with clinical outcome in fanconi anemia. European Fanconi Anemia Research Group. Blood 2000;96:4064–4070.
141 Joenje H, Arwert F, Kwee ML, Madan K, Hoehn H: Confounding factors in the diagnosis of Fanconi anaemia. Am J Med Genet 1998;79:403–405.

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Author Index

Abeysinghe, S.S. 17
Alberici, P. 149
Antoccia, A. 191

Batzer, M.A. 104
Bergoglio, V. 35

Callinan, P.A. 104
Chuzhanova, N. 17
Cooke, M.S. 53
Cooper, D.N. 17

Day, J.W. 67
Dick, K.A. 67

Evans, M.D. 53

Fodde, R. 149

Hoehn, H. 218

Ju, Z. 84

Kalb, R. 218
Kobayashi, J. 191
Komatsu, K. 191
Kuttler, F. 171

Loeb, L.A. 206
Lupski, J.R. 1

Magnaldo, T. 35
Mai, S. 171
Margolis, J.M. 67
Matsuura, S. 191

Nanda, I. 218
Neveling, K. 218
Nojima, H. 131

Ozgenc, A. 206

Qi, W. 116

Ranum, L.P.W. 67
Rudolph, K.L. 84

Schindler, D. 218
Stankiewicz, P. 1

Tauchi, H. 191

Yu, H. 116

Subject Index

Adenomatous polyposis coli (APC) protein 149
Aging 84, 206
Alternative splicing dysregulation 71
Alu sequences 3, 23, 109
Alzheimer's disease 59
Amyotrophic lateral sclerosis 59
Anaphase-promoting complex/cyclosome (APC/C) 117
Aneuploidy 124, 160
Angelman syndrome (AS) 6
Apoptosis 89, 182
Array comparative genomic hybridization (aCGH) 7, 151
Ataxia telangiectasia mutated (ATM) protein 133, 196, 228
ATM and Rad3-related (ATR) protein 138, 200, 228
ATR-Seckel syndrome 138
Autoimmune disease 58
Azoospermia c (AZFc) 7

Bone marrow failure 224
Budding uninhibited by benzimidazole (Bub) proteins 119, 159

Cancer 17, 40, 90, 116, 160, 171, 228
  predisposition 126, 133, 150, 192, 207, 219
Cardiovascular disease 62
ß-Catenin 153
Charcot-Marie-Tooth type 1 disease (CMT1A) 4
Checkpoint kinase
  protein 1 (CHK1) 141
  protein 2 (CHK2) 143
Chi (χ) elements 29
Chromatin remodeling 174, 230
Chromosome
  breakpoint junctions 19
  instability (CIN) 116, 132, 149, 193, 219
  segregation 117, 156
Chronic myeloid leukemia (CML) 5
c-Myc 171
Cockayne syndrome 45
Colorectal cancer 124, 150
Copy-number polymorphism (CNP) 7

Deletions 2, 17, 108, 111, 150, 209
Dent's disease 110
DeSanctis-Cacchione syndrome 43
Diabetes 62
DiGeorge/velocardiofacial (DGS/VCFS) syndrome 6
DNA
  damage and repair 35, 53, 89, 133, 139, 193, 226
  microhomologies 18
  mitochondrial 56
  non-B structures 21
  oxidation 54
  rearrangements 1, 17, 176, 209
  replication 28, 139, 215, 227
  secondary structures 22

strand breaks 18, 26, 132, 178, 198, 210, 221
Z-DNA 21
Duplications 2, 18, 150
Dyskeratosis congenita (DKC) 89

Emery-Dreifuss muscular dystrophy 6
Epigenetic modifications 125, 229
Extrachromosomal elements 177

Fanconi anemia (FA) 218
genes 226
Fluorescence in situ hybridization (FISH) 9, 151
Fragile-X-associated tremor/ataxia syndrome (FXTAS) 74
Friedreich's ataxia (FA) 59

Gene
amplification 176
conversion mediated deletion 111
Genome
disorders 1
instability 95, 171, 208, 218
locus-specific 176
transposable elements 104
Gross rearrangement breakpoint database (GraBD) 19

Hemophilia A 6, 110
Human gene mutation database 110
Hunter disease 6
Huntington's disease (HD) 59
like 2 (HDL2) disorder 77
8-Hydroxy-7,8-dihydro-2′-deoxyguanosine (8-OH-dG) 55

Immunodeficiency
role of NBS1 198
X-linked agammaglobulinemia (XLA) 112
Inversions 6, 150, 209

Kinetochore 120, 158

Large-scale copy-number variation (LCV) 7
Leukemia 5, 92, 192, 224
LINE-1 (L1) retrotransposons 105
Low-copy repeats (LCRs) 2
Lymphomas 181, 192

Microtubules 117, 155
Mitosis 117
Mitotic
arrest deficiency (Mad) proteins 119, 174
checkpoint complex (MCC) 121
Mosaic variegated aneuploidy syndrome (MVA) 126
Mouse models 68, 75, 86, 125, 161, 180, 232
Multiplex ligation-dependent probe amplification (MLPA) 9
Muscular dystrophy 108
Myelodysplastic syndrome (MDS) 92
Myotonic dystrophy
type 1 (DM1) 68
type 2 (DM2) 69

Neurodegenerative conditions 59
Nijmegen breakage syndrome 191
Nucleotide excision repair (NER) 35
Nucleus organization 179

Oncogene 171
Oxidative damage 53

Palindromes 22
Pancreatic cancer 228
Parkinson's disease (PD) 59
p53 89, 133, 214
Prader-Willi syndrome (PWS) 6
Protein kinases 131
Pulsed-field gel electrophoresis (PFGE) 9

Radiosensitivity of NBS cells 193
Reactive oxygen species (ROS) 53, 92, 231
Recombination
Alu-Alu 24, 111
hotspots 3, 18
immunoglobulin heavy-chain class switch 29
non-allelic homologous (NAHR) 2, 18, 111
non-homologous 24, 178
V(D)J 26
RecQ helicase family 206, 227

Repeat expansion
human diseases 67
RNA-binding proteins 71
RNA gain-of-function mechanism 70
Replicative senescence 208
Representational oligonucleotide microarray analysis (ROMA) 7
Retrotransposable elements 104
Retrotransposition 107
Reverse transcriptase 85, 105
Rheumatoid arthritis (RA) 58

Sequence transduction through retrotransposition 109
Skin photosensitivity 40
Smith-Magenis syndrome (SMS) 4
Somatic reversion 233
Sotos syndrome 4
Spindle 116, 158
Spinocerebellar ataxia (SCA) 76
Stem cells 84
SVA elements 112
Systemic lupus erythematosus (SLE) 58

Target-primed reverse transcription (TPRT) 105
Telomerase 84, 179
Telomeres 84, 214
Topoisomerase 30
Transcription-coupled repair (TCR) 36
Translin 30
Translocations 17, 27, 150, 209
Transposable elements 104
Trichothiodystrophy (TTD) 46
Tumor suppressor 124, 149

UV irradiation 36, 139

Werner syndrome (WS) 206
Williams-Beuren syndrome 6
Wnt signaling 153
WRN helicase/exonuclease 211

Xeroderma pigmentosum 39
X-linked genetic disease 107